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ANTIMICROBIAL WOUND DRESSING

FINAL, PHASE I REPORT

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The main objective of Phase I of this SBIR project was to demonstrate the feasibility for successful development of an antimicrobial wound dressing based on a hydrogel sustained release matrix. This goal has been achieved.

In vitro studies of drug diffusion have demonstrated that BIOTEK wound dressings can release antimicrobial agents for up to one week. Wound dressings based on polyvinyl alcohol, polyhydroxyethyl methacrylate, polyacrylamide and polyethylene oxide have been evaluated. Release rates are dependent upon water content, degree of hydrogel crosslinking, concentration of plasticizer, polymer molecular weight, degree of hydrolysis of polyvinyl alcohol, and solubility of the antimicrobial agent. A wide range of release rates may be achieved by selection of the proper hydrogel matrix.

Polyvinyl alcohol based hydrogels containing either tetracycline free base or chlorhexidine diphosphanilate were efficacious in vivo in a wound model with an established Streptococcus pyogenes infection. In addition chlorhexidine diphosphanilate hydrogels were efficacious against established staphylococcus aureus infections and mixed infections containing both organisms.

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FOREWORD

In conducting the research described in this report, the investigator(s) adhere to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals (DHEW Publication No. (NIH) 85-23, Revised 1985).

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For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

I. SUMMARY

The main objective of Phase I of this SBIR project was to demonstrate the feasibility for successful development of an antimicrobial wound dressing based on a hydrogel sustained release matrix. This goal has been achieved.

In vitro studies of drug diffusion have demonstrated that BIOTEK wound dressings can release antimicrobial agents for up to one week. Wound dressings based on polyvinyl alcohol, polyhydroxyethyl methacrylate, polyacrylamide and polyethylene oxide have been evaluated. Release rates are dependent upon water content, degree of hydrogel crosslinking, concentration of plasticizer, polymer molecular weight, degree of hydrolysis of polyvinyl alcohol, and solubility of the antimicrobial agent. A wide range of release rates may be achieved by selection of the proper hydrogel matrix.

Polyvinyl alcohol based hydrogels containing either tetracycline free base or chlorhexidine diphosphanilate were efficacious in vivo in a wound model with an established Streptococcus pyogenes infection (see Table 1). In addition chlorhexidine diphosphanilate hydrogels were efficacious against established staphylococcus aureus infections and mixed infections containing both organisms (see Table 2).

II. INTRODUCTION

A. Objectives

The overall objective of the project is to develop a soft, conforming wound dressing containing a broad spectrum antimicrobial agent for the initial treatment of avulsive wounds and burns. The wound dressing should generate a sterile site and protect the site for at least 7 days or until definitive care can be initiated. The objectives of the Phase I SBIR studies are to demonstrate the feasibility of successful development of the product and to develop the experimental approach and methodology necessary for further research.

Specific Aims

1) Prepare sustained release antimicrobial hydrogels. Several polymers will be evaluated including polyvinyl alcohol, poly-hydroxyethyl methacrylate, and polyacrylamide. Two broad spectrum antimicrobial agents, chlorhexidine diphosphanilate and tetracycline, will be incorporated into the sustained release matrixes.

2) Characterize the materials by in vitro drug release kinetics under sink conditions, hydration properties, and morphology as revealed by scanning electron microscopy (SEM).

3) Develop and prepare prototype wound dressings using the most promising sustained release matrixes.

4) Evaluate the in vivo efficacy of prototype wound dressings in an animal model.

5) Demonstrate sustained drug release from the prototype wound dressings in an in vivo animal model and compared to the in vitro results.

3. Background

A combat injury requires immediate attention to stop the bleeding and generate a sterile site for wound healing. A delay of only 4 hours may be significant and result in an established infection with consequent therapeutic difficulties (Burke, 1986; Rodheaver, et al., 1974). The injured soldier may also be required to continue fighting or be able to evacuate the area under his own power. For less severe injuries, sustained-release drug delivery in military medicine would allow personnel to perform vital combat functions after receiving injuries which would otherwise require removal from the battlefield.

Infection of the wound site is the most common medical complication after the initial blood loss and shock symptoms have been stabilized. Combat wounds are characterized by a high incidence of infection. This may be due to the presence of devitalized tissue, the presence of foreign bodies in the wound, and/or because there is an unavoidable delay in treating wounds in a combat situation. The immediate treatment may require cleansing the wound area, prior to bandaging. The effectiveness of this procedure depends both on the skill of the paramedic and on the field conditions. The infection process is peculiar to the microorganisms causing the infection and to the mode of action of the antibiotics or antiseptics which are used to combat the infection. However, the advantage of the continuous presence of the anti-infective agent at the wound site has been demonstrated for many wound dressings, and also for systemic antibiotic (p.o., i.v., etc.) regimens used to control local infections. It should be noted that in the treatment of avulsive wounds and burns, shock and compromised peripheral circulation, compound the difficulties of systemic therapy (Gelin and Border, 1970).

In the usual clinical setting it is desirable to institute therapy with an appropriate anti-microbial agent by isolating the pathogen(s) and determin-

ing sensitivity (Sande and Mandell, 1980). While even in the ideal situation this approach is not uniformly utilized (c.f. Kunin and Edelman, 1978), it is clearly impossible in field combat conditions. Therefore, broad spectrum antibiotics and antiseptics may be particularly useful for initial management of combat wounds.

A high concentration of antimicrobial is required for the initial microbial challenge. However, time of contact is also important, and the relative therapeutic effectiveness of intermittent and continuous dosage regimens for antibacterial agents is uncertain (Toothaker, Welling and Craig, 1982). Correlations have been attempted with various pharmacokinetic parameters such as the maximum plasma concentration and the time during which the drug level exceeds the minimum inhibitory concentration. Too high a concentration of an antiseptic may cause tissue cell damage and too short a time period may not allow contact during the life cycle of the bacterial cell at which it is most susceptible to biocidal agents. Thus a time release of antiseptic agents at the local site should be useful. Specific studies (e.g. Georgiade and Harris, 1973, for povidone iodine) have shown significant advantages of multiple dosing regimens for antiseptics.

Chlorhexidine diphosphanilate is an antiseptic agent in which both ions have bactericidal activity. This is a proprietary product which is available only from Westwood Pharmaceuticals (Bristol Laboratories). Chlorhexidine is bacteriocidal to both gram-positive and gram-negative bacteria, although it is not as potent against the latter. In a 4% aqueous solution as a surgical scrub, it decreases the cutaneous bacterial population more than either hexachlorophene or povidone iodine. Chlorhexidine is used for the preoperative preparation of both surgeon and patient for the treatment of burns and the irrigation of wounds and surgical infections.

Phosphanilic acid (p-aminobenzenephosphonic acid) is not readily available, nor is there extensive literature on this compound. Thayer, Magnuson

and Gravatt (1953) describe the antibacterial action of several compounds, including phosphanilic acid. Kanitkar and Bhide (1947) give some data on the effectiveness of phosphanilic acid against Staphylococcus aureus. Lee, et al. (1980) describe the bioavailability and metabolism of phosphanilic acid. In general the activity is similar to the sulfanilamides.

Tetracycline is the prototypic agent, although it was the third discovered, of a class of antibiotics isolated from Streptomyces. This class of antibiotics possess a wide spectrum of activity against gram positive and negative organisms and both aerobic and anaerobic pathogens (Sande and Mandell, 1980). The broad spectrum of anti-microbial activity contributed to the early popularity and indiscriminate use of these agents. Currently this class is rarely the first drug of choice but is frequently used for resistent organisms (Sande and Mandell, 1980).

Hydrogels are crosslinked three dimensional polymeric arrays which can imbibe large quantities of water. In the hydrated state these materials are moist, pliable and can conform to irregular surfaces. For more than two decades these materials have been studied as nonthrombogenic surfaces (Kearney, et al., 1975; Ratner and Hoffman, 1975; Kronick, 1983), as drug delivery systems (Kim, et al., 1980), for encapsulating of living cells (Lim, 1984) and for other biomedical purposes (Schacht, 1984). A given monomer can produce radically different hydrogels by varying the ratio of monomer and crosslinking agent, type of crosslinking agent, and degree of dilution. Some of these parameters have been reviewed by Schacht (1984). For example increasing the degree of crosslinking which reduced pore size can reduce diffusion coefficients by two orders of magnitude.

C. Military Relevance

While rapid transport of combat casualties to a site for definitive treatment is a major goal in military medicine, it cannot be assumed nor as-

sured. In some cases a casualty may be required to continue his duties and evacuate himself. A period of seven days between injury and definitive care might easily occur. In such cases the initial treatment applied in the field is crucial. Two types of wounds of military importance are particularly prone to infection, avulsive wounds and burns. A wound dressing which incorporates an antimicrobial agent might allow a slowly evacuated casualty to begin definitive care with a sterile or nearly sterile wound.

III. ACCOMPLISHMENTS

A. Methods

1. Preparation of Hydrogels

a. Polyvinyl Alcohol

Polyvinyl alcohols (PVA's) were obtained from Air Products and Chemicals, Allentown, Pennsylvania. Physical characteristics of PVA's utilized in this project are presented in Table 3. PVA 10% (w/v) was suspended in distilled water, heated to approximately 85°C for PVA 523 or 95°C for PVA 350 and 425, and mixed for at least 30 minutes to prepare solutions. Plasticizers and crosslinking agents were added to cool solutions and their concentrations were calculated based on dry weight of PVA. Antimicrobial agents were added to the complete matrix, and films were cast in glass molds. Films were dried at room temperature and stored in aluminum foil. Drug concentrations are expressed as percent (w/w) of total matrix.

b. Polyethylene Oxide

A polyethylene oxide hydrogel, radiation crosslinked and polymerized, was obtained from the manufacturer, Nepara, Harriman, NY. The material is 4% (w/w) polymer 96% water and is layered over and under a porous polyethylene sheet for reinforcement.

c. Polyacrylamide

Acrylamide and N,N'-methylene bisacrylamide were obtained from Sigma Chemical Co., St. Louis, Mo. A 4% (w/v) hydrogel with 0.1% crosslinker was polymerized in 50 mM tris hydrochloride buffer, pH 7, using 3 mM ammonium persulfate as a free radical source. Films were cast in open molds under a

nitrogen atmosphere. As in pilot studies dried films were very brittle and could not be manipulated. Therefore hydrated gels were utilized for testing.

Neither tetracycline nor chlorhexidine diphosphanilate were stable in the presence of ammonium persulfate. Free radicals apparently oxidized the antimicrobial agents and when the drugs were present during polymerization the films were dark brown.

d. Polyhydroxyethyl Methacrylate

2-hydroxyethyl methacrylate (opthalmic grade, HEMA) and ethylene glycol dimethacrylate (EGDMA) were obtained from Polysciences, Warrington, Pa. Hydroquinone inhibitors were removed by ion exchange chromatography (60 ml/hr) on De-Hibit 100 resin (Polysciences), and the material used without further purification. The ratio of EGDMA to HEMA was varied from 0.5 to 2% (v/v), and hydrogels were prepared with 40 to 20% (v/v) water content. Free radical polymerization was initiated with either 0.6 mM ammonium persulfate (Fisher) or 7.84 mmoles 2,2'-azobisisobutyronitrile (Polysciences) per ml of monomer.

Solutions were purged with zero grade nitrogen and cast in open molds under a nitrogen atmosphere or in molds prepared from glass plates separated by 0.1 or 1 mm medical silicone sheet (Dow) spacers. Polymerization was conducted at 50-60°C. These methods are based on those of deLeede, et al (1986). The open molds gave gels approximately 1 cm thick, but they were not homogeneous. The thin molds were removed from the oven as soon as jelling was evident, the glass molds were opened and the film was transferred to a 400 ml glass beaker with 250 ml of distilled water and boiled for 10 minutes to facilitate the removal of unreacted monomer. The films were then removed from the boiling water and allowed to stand in 500 ml of 37°C distilled water for 72 hours.

When the water content exceeded approximately 40%, opaque non-homogeneous gels were obtained. Also, with increasing water content, the polymerization

was inhibited or occurred in only localized areas giving non-homogeneous amorphous masses of polymer mixed with unreacted monomer solution. In all preparations gels containing 2% EGDMA were more rigid than those containing 1%. Areas of localized polymerization which gave rise to "popcorn" polymers were observed at the 1 and 2% concentration of crosslinker. Decreasing the EGDMA concentration to 0.5% prevented this problem. Clear homogeneous gels were obtained with 33% total water and 0.5% EGDMA and occasionally 1.0% EGDMA.

2. In Vitro Diffusion

a. Apparatus

In previous work conducted in this laboratory (Gay, et al., 1984) the kinetics of drug diffusion from sustained release matrices were monitored after immersing the test material in a quantity of water or buffer, usually 40 or 300 ml, which would insure that drug concentration did not exceed 10% of saturation. Under these sink conditions drug release occurs from all surfaces of the matrix. This differs from the geometry of the in use wound dressing and the method does not permit evaluation of prototype wound dressings. Therefore a new in vitro test system was developed. The wound dressing or sustained release matrix is mounted in a 20 mm flow through Franz diffusion cell (Model FDC 200, Crown Glass, Somerville, New Jersey). Up to nine cells can be mounted in a drive console (Model FDCD-9, Crown Glass) with magnetic stirrers to insure proper mixing of the perfusate. An eight cell drive console and the flow through cell are diagrammed in Figure 1. Saline (0.9% w/v is perfused through the system with a multiple channel cassette peristaltic pump (Manostat, New York, NY). Perfusion rate, 5-20 ml/hr, is selected so that drug concentration does not exceed 20% of saturation. Therefore, diffusion is studied under sink conditions. A Fractomat automatic fraction collector (Buchler, Fort Lee, New Jersey) collects the perfusate. The system is controlled by Chrontrol Model CD-4 timer (Lindberg Enterprises, San Diego, Calif). The perfusate and the Franz diffusion cell are maintained at 35°C,

external skin temperature, by a Masterline circulating water bath Model 2095 (Forma Scientific, Marietta, Ohio). Drug concentration in the perfusate is determined by u.v. spectroscopy. In vitro diffusion studies were conducted in duplicate or triplicate, and control hydrogels, identical to test gels but without drug, were run in parallel.

b. Wound Dressing

In order to hold hydrogels in Franz diffusion cells, a support was designed as part of the preliminary work in preparation of the proposal for this contract. Dried hydrogels are mounted in 1 mm vacuum thermoformed depressions in 30 mil adhesive backed occlusive closed cell polyethylene foam (Avery, Painsville, Ohio). A separate compartment contained distilled water or saline to hydrate the film when required. A membrane prepared from polyester mesh with approximately 30 μm pores covers the diffusion surface. This membrane is only required when delicate hydrogels which may fragment or disintegrate are tested. The hydrogel support is diagrammed in Figure 2. While this system was designed to support the hydrogel in the in vitro test system, it may also serve as a model for the prototype packaging of the wound dressing. This design is based on BIOTEK's patented DermaPatch transdermal delivery system.

c. Assay

In order to determine the drug release kinetics of the hydrogels it was necessary to determine solubilities and to construct standard curves for the two antimicrobial agents, chlorhexidine diphosphanilate and tetracycline hydrochloride. Chlorhexidine diphosphanilate standards were prepared in distilled water in the concentration range 0.7 to 16.8 μg/ml. An absorption maximum was observed between 245 and 250 nm, and 250 nm was selected as the wavelength for assay. A linear standard curve (Figure 3) with a least squares correlation coefficient of 0.9997 and a factor of 19.0 μg/ml O.D. ($\pm$ 0.4

S.E.M.) was obtained. A standard curve based on the diacetate salt gave a factor of 25.2 μg/ml O.D. (± 0.9 S.E.M.) with a correlation coefficient of 0.9999 (Figure 3). On a weight basis the factor for the diacetate salt was 133% of that for the diphosphanilate. However, the molar extinction coefficients are 24825 for the diacetate and 44825 for the diphosphanilate salts. Therefore both chlorhexidine and diphosphanilate ions absorb at this wavelength, and each ion contributes approximately equally. Saturation was estimated by mixing about 50 mg of chlorhexidine diphosphanilate with 5 ml of distilled water or 0.9% saline for 48 hours at room temperature and quantitating drug concentration in a filtrate. Chlorhexidine diphosphanilate was soluble to the extent of 664.2 μg/ml of distilled water (± 7.9 S.E.M.) and 978 μg/ml of saline (± 54 S.E.M.).

Similar studies were conducted with tetracycline hydrochloride. The standard curve was constructed over the concentration range 1 to 60 μg/ml, and approximately 250 mg of material was mixed with 3 ml of solvent for determination of saturation levels. The assay was conducted at 380 nm. The standard curve was linear with a least squares correlation coefficient of 0.9997 (Figure 4). The calculation factor was 54.5 mg/ml O.D. (± 2.4 S.E.M.). Saturation was 97.3 mg/ml (± 0.8 S.E.M.) in distilled water with 111.8 mg/ml (± 0.6 S.E.M.) in normal saline. These values are quite close to literature values of 100 mg/ml (Harvey, 1980).

3. Physical Characterization

a. Scanning Electron Microscopy (S.E.M.)

Discs 7 mm in diameter were punched out of the matrixes and dried over desiccant for several days. The samples were sent to a commercial laboratory for S.E.M. evaluation.

b. Hydration

Discs, 0.25 inches in diameter were cut from the films with a paper punch. Initial weight was determined to 0.1 mg on an analytical balance, and initial thickness was determined with a Yuasa (Carlstadt, N.J.) dial micrometer. Discs were placed in approximately 2 ml of either distilled water or 0.9% saline at room temperature. At different times samples were blotted dry and measured. Experiments were conducted in triplicate.

4. In Vivo Efficacy

a. Antimicrobial Sensitivity Tests

Four bacterial strains were obtained from the American Type Tissue Collection, Staphylococcus aureus strains 29996 and 29247 and Streptococcus pyogenes strains 19615 and 14289. The objective was to select a single strain that would be appropriate for the in vivo tests. Therefore the sensitivity of these bacteria to chlorhexidine diphosphanilate and tetracycline HCl was determined. Lyopholyzed bacterial samples were hydrated with trypticase soy broth (Gibco Laboratories, Madison, Wisconsin) and transferred to this medium for growth at 37°C for 24 hours. Tetracycline HCl and chlorhexidine diphosphanilate were prepared in trypticase soy broth by serial dilution and then autoclaved for 15 minutes. These tubes were innoculated with the four strains and incubated for 28 hours at 37°C.

It was noted that the more concentrated tetracycline tubes darkened somewhat during sterilization. Since this may indicate drug degradation, dry tetracycline base (Sigma Chemical Company, St. Louis, MO) was sterilized in a nitrogen atmosphere and no degradation (color change) was apparent. Sterile drug was then serially diluted in trypticase soy broth. These tubes were then innoculated with the four bacterial strains and incubated for 24 hours at 37°C.

Sensitivity was also evaluated for the formulated antimicrobial agents by a disc diffusion assay. Bacteria were streaked onto plates of Standard Methods Agar (Gibco), and a 7 mm diameter disc of control or drug loaded matrix was placed in the center. Plates were incubated for 24 hours at 37°C and areas of inhibition measured manually.

b. Animal Model

In vivo efficacy tests were based on the animal model described by Setterstrom, et al. (1985) which utilizes the microbial quantitation method of Williamson and Klingman (1965). Male Hartley strain guinea pigs, 300 to 350 g, were obtained from Charles River Laboratories (Wilmington, MA). Hair was clipped from the animal back, and a commercial depilatory (Nair™) was applied to remove remaining fur. Animals were anesthetized with sodium pentobarbital and the surgical site cleaned with Betadine™. A full thickness skin flap was taken from the intrascapular area to leave a 2.5 x 2.5 cm wound exposing subcutaneous fat and muscle. A 50μl aliquot of a bacterial culture was placed in the wound. The surface was covered with sterile polyethylene and secured with an adhesive backed, closed cell, occlusive polyethylene foam (Avery). The dressing was protected with a double layer of orthopedic stockinet secured with surgical tape.

Three days following infection, animals were anesthetized with sodium pentobarbital, 30 mg/kg, and the dressings removed. A hydrated antimicrobial wound dressing or control dressing prepared from matrix with no drug was placed over the wound surface and secured with occlusive foam and orthopedic stockinet. Following 6 days of therapy animals were sacrificed with carbon dioxide and wound dressings removed. A sterile 2.33 cm^2 chamber prepared from a Vacutainer™ holder (Becton Dickinson, Rutherford, NJ) was held firmly against the wound surface. A 1 ml aliquot of .05% Triton X-100 in 40 mM phosphate buffer, pH 7.4 was transferred into the chamber, and the surface

mixed with a sterile flattened glass rod. The fluid was transferred to a sterile test tube, the procedure repeated with an additional 1 ml aliquot, the samples combined and stored on ice. All of the procedures were conducted aseptically, and each experimental group was 5 animals.

Within 3 hours the samples were appropriately diluted, an aliquot was added to 10 ml of Standard Methods Agar, and a pour plate was prepared. Colonies were counted manually following incubation at 37°C for 24 hours, and the number of Colony Forming Units (C.F.U.) per square cm of wound surface was calculated.

5. Preparation of Microcapsules

Chlorhexidine diphosphanilate and tetracycline hydrochloride were microencapsulated using BIOTEK's proprietary air suspension coating process (Nuwayser, 1986). Chlorhexidine diphosphanilate was coated with poly-L(-)-lactide to a drug loading of 69%. the distribution of microcapsules size is shown in Table 4. Tetracycline hydrochloride was coated with cellulose triacetate since a biodegradable polymer is not required for a topical product. A 10% polymer coat was applied to 8.4 g of starting material with a final yield of 87.6%. The desired size range was 100 to 600 microns. The size range of the product was analyzed by sieve analysis, and the results are presented in Table 5. Nearly 97% of the material is in a size range suitable for incorporation into wound dressings.

B. Results

1. In Vitro Diffusion

a. PVA Hydrogels

1) Types of PVA

Initial studies were conducted with PVA hydrogels, and the effect of molecular weight and degree of hydrolysis on release was investigated. Chlorhexidine diphosphanilate release was nearly complete in 10 hours (Figure 5) from a matrix based on PVA 523 plasticized with 20% glycerol. The matrix was nearly completely dissolved at the conclusion of the study (40 hour). Similar 20% glycerol plasticized matrices prepared from PVA's 425 and 350 release drug more slowly. Under these sink conditions approximately 50% of the drug load was released at 40 hours and release was continuing. No significant differences were observed between PVA 350 and 425 during initial release, although teminal release was slightly greater from the 425 based matrix and drug concentration in perfusate was greater for PVA 425, 16 μg/ml, than for PVA 350, 8 μg/ml. Very similar results were obtained when these matrices contain 20% tetracycline as the free base were tested (Figure 6). With this drug there was significantly greater release rate from PVA 425 based matrix compared to PVA 350. Nearly 100% release was observed at 15 hours for PVA 523 and the matrix was nearly completely dissolved at 48 hours. PVA 425 based matrix released nearly all of the drug by 48 hours while at this time 82% was released from the PVA 350 based matrix. Drug concentration in the final samples at 48 hours was 29 μg/ml for both PVA 425 and 350. With these matrices the most rapid release was observed when tetracycline hydrochloride was incorporated into the matrix at 20% w/w concentration (Figure 7).

The release rates observed in this study correlate with the physical properties of the PVA's. The most rapid release was observed with the least

hydrolyzed PVA, 523, which is the most soluble in water. The slowest release was observed with the fully hydrolyzed, least water soluble PVA, 350. Molecular weight of the polymer had little observable effect. The molecular weight's of PVA's 425 and 523 are both in the range 77,000 to 79,000 daltons and their release rates are very different. PVA 350 which had release characteristics similar to PVA 425 has a molecular weight in the range of 106,000 to 110,000 daltons.

2) Drug Loading

The effect of drug loading on release rates was investigated using tetracycline hydrochloride and chlorhexidine diphosphanilate in a PVA 425 matrix plasticized with glycerol, 20% (w/v). As shown in Figure 8, the amount of tetracycline-HCl released during the initial 6 hours was clearly dependent upon the drug concentration in the matrix. Release rates declined markedly following the 1 hour observation period, and no difference was observed in the release rates from 23 and 12% (w/w) drug loaded matrices. As shown on the cumulative curve (Figure 9) all of the drug was released from the two lower concentrations, but only 60% of the higher loading was released in this time period. In a similar study, chlorhexidine diphosphanilate cumulative percent release was greatest at 1% (w/w) drug loading (Figure 10). Little difference was seen in the first 9 hours between 11 and 20% (w/w) loaded samples, but the rate for the 11% loaded sample accelerated at that time. At 24 hours nearly all of the drug was released from 1 and 11% (w/w) loaded samples, and only 45% had been released from the 20% (w/w) loaded matrix.

3) Drug Forms

In the previous studies it was observed that the more soluble preparations, both matrix and drug, released drug more rapidly than less soluble formulations. This observation was extended by studying the release rate of different forms of tetracycline from PVA 425 matrix plasticized with glycerol 20%

(w/w). As shown in Figure 11, the hydrochloride, the most soluble form, was released most rapidly. The free base form of the drug released much slower, and complete release under sink conditions was reached in about 6 days. Under these sink conditions tetracycline concentration in the perfusate was maintained above 9 μg/ml throughout the 6.5 day study. When microcapsules containing tetracycline-HCl were incorporated into the matrix drug release rate was reduced further, and only about 68% of the total drug load was released in 6 days. Similar results were obtained with chlorhexidine diphosphanilate (Figure 12). This drug is only 0.7 to 0.9% as soluble as tetracycline-HCl (Section III.A.2.) and microencapsulation had a much greater effect on its release rate. Only 8% of the microencapsulated chlorhexidine diphosphanilate was released in 2 days, while in the same time period approximately 44% of the microencapsulated tetracycline-HCl was released. The kinetics of release from chlorhexidine diphosphanilate microcapsules incorporated into the PVA matrix were clearly zero order which is characteristic of this type of microcapsule.

4) Matrix Variations

The characteristics of PVA 425 matrix were altered by crosslinking the polymer with glutaraldehyde, altering the quantitation of plasticizer, altering the degree of crystallinity by heating, and finally by blending PVA's of different molecular weights and degrees of hydrolysis. Preliminary studies presented in the proposal demonstrated that crosslinking the polymer decreased the rate of drug release. This observation was confirmed by comparing <u>in vitro</u> release rates of chlorhexidine diphosphanilate from glycerol plasticized PVA 425 hydrogels with and without glutaraldehyde. As shown in Figure 13, 5% (v/w) glutaraldehyde significantly reduced the release rate when the drug was suspended in the matrix. Without the crosslinker approximately 45% of the drug was released in 24 hours while the matrix with 5% glutaraldehyde released less than 15% of the drug load in this time period. Similarly when the drug

was present as a suspension of microcapsules in the matrix, 1.8% glutaraldehyde reduced the rate of chlorhexidine diphosphanilate release (Figure 14).

The rate of release of chlorhexidine diphosphanilate was apparently dependent upon plasticizer concentration. Increasing the glycerol concentration from 20% (w/w) of PVA to 40% significantly reduced the rate of drug release (Figure 15).

PVA 425 films plasticized with 20% glycerol and containing approximately 40% drug were heated at 100°C for varying periods of time to increase the degree of crystallinity of the films. Blanks containing no drug were clear and hardened significantly following heating for one or more hours. The tetracycline gels which initially were yellow and very pliable darkened slightly after 1 hour at 100°C but were still pliable. With continued heating, color changed progressively to brown then black, and films hardened significantly. Gels containing chlorhexidine did not change color with heating but did become less pliable.

Figure 16 shows that there was little change in the release kinetics of chlorhexidine diphosphanilate when the films were heated for up to 24 hours. The decreased level of the plateau following complete release may indicate slight degradation. As shown in Figure 17 there was significantly reduced maximum release of tetracycline after heating for only 1 hour. This was more prominent after 24 hours. This probably reflects significant drug degradation.

Since significant differences in release rates were observed with different PVA's, blends of these materials were tested to determine if intermediate release rates could be obtained. Selections of PVA's with molecular weights in the range 77,000 to 79,000 daltons, 523 (87-89% hydrolyzed) and 425 (95.5-96.5% hydrolyzed), were blended to give matrices containing 20%

glycerol, 40%, 53%, or 80% PVA 523, and the remainder was PVA 425. Adding PVA 425 to the PVA 523 significantly reduced rate of both chlorhexidine diphosphanilate (**Figure 18**) and tetracycline (**Figure 19**) release in a concentration dependent manner.

b. Poly-HEMA Gels

Wound dressings containing approximately 10 mg of chlorhexidine diphosphanilate or tetracycline free base were prepared from several poly-HEMA hydrogels and tested for in vitro drug release. Since the antimicrobial agents were not stable in the presence of the free radical initiator and heat, they could not be suspended in the hydrogels during polymerization. A wound dressing similar to that shown in **Figure 2** was prepared with hydrated hydrogels with dry drug in the upper compartment. The polyethylene membrane was omitted since the dressing was to be used immediately. Chlorhexidine diphosphanilate release was dependent upon both the water content of the gel and the degree of crosslinking (**Figures 20 and 21**). Diffusion was slowest when hydrogels contained less water, 20%, and the higher, 1%, concentration of crosslinking agent, EGDMA. After an initial burst nearly linear release of approximately 0.2 mg/hour was observed from 8 to 48 hours for gels containing 20% water (**Figure 20**). Rate of release increased significantly when EGDMA concentration was reduced to 0.5%, and increased dramatically in poly-HEMA gels containing 40% water. Release from gels with 40% water occurred in multiple bursts. The results are identical if the percent release (**Figure 21**) is compared to amount released (**Figure 20**).

With tetracycline the most significant decrease in release rate occurred when concentration of crosslinker, EGDMA, was reduced from 1.0 to 0.5% (**Figure 22**). There was a slight decrease in release when hydrogel water content was increased from 20 to 30%, and this was clearer when percent release curves were compared (**Figure 23**). Tetracycline release appeared to occur in multiple bursts in all samples and a repeat of the experiment confirmed this observa-

tion. This pattern may be caused by uneven swelling of the boundary of the film which restricts the release of the drug until equilibrium is achieved. The differences between release characteristics of these two drugs may be due to pH differences or drug interactions with the polymer.

c. Polyacrylamide Gel

Wound dressing contain 20% (w/w) chlorhexidine diphosphanilate as microcapsules were prepared using polyacrylamide gel, and in vitro release rates were evaluated. The results of this study are shown in **Figure 24.** Following a 1 hour lag period there was a burst release of nearly 50 μg of chlorhexidine diphosphanilate during a 2 hour period. During the subsequent 23 hours, drug release rate was nearly constant at slightly greater than 2 μg per hour. The lag period probably reflects the time required to hydrate the microcapsules and then diffuse drug into the water phase of the hydrogel. PVA 425 matrix with chlorhexidine diphosphanilate microcapsules released drug at nearly 10 times greater rate (Section III.B.3).

d. Polyethylene Oxide Gel

A polyethylene oxide hydrogel, radiation crosslinked and polymerized, composed of 4% polymer and 96% water was obtained commercially. This material was evaluated in vitro as a wound dressing matrix using tetracycline. One series of gel samples was soaked in a saturated saline solution of tetracycline-HCl for 24 hours to load the gel. Disks of this material were rinsed in saline and blotted, placed on the adhesive foam backing material, covered with fine mesh (40 μm) polyester, and placed on the flow through diffusion cell. Other samples were prepared by placing either tetracycline-HCl or tetracycline-free base on the adhesive foam backing, covering the dry drug with a disk of polyethylene oxide hydrogel, covering the gel with polyester fabric, and mounting the dressing on the flow through diffusion cell. The results are shown in **Figure 25.** There was an initial burst observed during

the first 4 hours with both the soaked gel and tetracycline-HCl. As expected the relatively insoluble free base provided nearly constant release throughout the study period. Characteristics of tetracycline release are shown in Table 6. The rate of release during the linear portion was slowest 47.5 μg/hr for the free base, fastest for the soaked gel, 136.4 μg/hr, and intermediate for the hydrochloride, 72.8 μg/hr.

Microcapsules were incorporated into polyethylene oxide based wound dressings as described for the free base and hydrochloride and tested. There was a significant lag period, 2 hours, preceding a period of first order release (Figure 26). Linear release was observed for the salt form and the smaller microcapsules following the 10 hour observation period and for the larger microcapsules following 20 hours. During the linear phase the rate of release was less than that observed for the hydrochloride and little difference was observed in the rates due to size 45.3 to 58.5 μg/hr (Table 6). The total amount of drug released in 24 hours reflected the initial burst and increased from 2.78 mg for the smallest microcapsules to 8.20 for the largest (Table 6). these values fall between those observed for the hydrochloride and the free base forms of the drug.

The differences in initial release and total release between the tetracycline-HCl soaked gel, the tetracycline-HCl powder loaded gel, and the free base loaded gel can be explained on the basis of amount of drug and solubility of the drug formulation. However, the microcapsule loaded gel data is more difficult to understand. Based on both total drug released and on percent drug release, the smaller microcapsules should have shown a shorter lag time and greater initial rate of release than the larger microparticles. The opposite was observed. This may be a result of the layered nature of the wound dressing prepared. The layers of small particles may have restricted access of the water to the microcapsules and resulted in localized areas of drug saturation. The closer packing of the smaller particles may result in a lower effective volume of saturated solution from which the drug diffuses into the body of the hydrogel.

2. Physical Characteristics

a. Scanning Electron Microscopy

The morphology of 20% glycerol plasticized PVA-425 matrices containing chlorhexidine disphosphanilate is shown in Figures 27 and 28. The cross section (Figure 27) shows an apparently interconnected mesh of channels. The surface clearly shows drug crystals imbedded in the polymer matrix. Similar morphology was observed with the 2% glutaraldehyde crosslinked matrix, with interconnected channels visible in cross section (Figure 29) and dry crystals present in the matrix (Figure 30). In addition there were a number of 2 to 5 μm pores in the surface which were not apparent in the uncrosslinked material.

b. Hydration

The initial thickness of the control PVA film and films containing suspension of drug was about 0.02 inches. Films containing microcapsules were approximately twice as thick. Results of swelling are presented in Table 7. Thickness was difficult to determine in both wet and dry states. Dry films were somewhat irregular, and when they contained microcapsules a range of 100% between films and within the same film was common. Wet films were much more consistent but also were quite soft and easily deformed. After 45 minutes of hydration the films without microcapsules had swollen from 160 to 170% of initial values. Films containing microcapsules had swollen much less, 100 to 130% of initial values. This difference probably reflects the initial difference in thickness and the possibility that the film was much thinner in regions between the groups of microcapsules than over the microcapsules. Diameters of the wet and dry discs were much easier to measure. As shown in Table 7 most of the swelling occurred within the first 45 minutes. There was little difference between the degree of swelling in either water or saline. No consistent difference in swelling properties could be attributed to either the microcapsules or the 1.5% glutaraldehyde.

The results of the hydration study are presented in Table 8. The control PVA/20% glycerol film increased in weight by 211% in both distilled water and saline. For all films glycerol appeared to be diffusing into the media. For all drug loaded films there was a somewhat higher degree of hydration in saline than in distilled water. Glutaraldehyde cross-linked films showed significantly less hydration than the others, and the difference between saline and water hydration was less pronounced for the cross-linked films.

The observed swelling was very similar to the pilot studies conducted in this laboratory (see Phase I proposal). However, the degree of hydration was significantly less, approximately 50%, than values obtained in the pilot study. In part this may be a result of loss of chlorhexidine diphosphanilate crystals from the films. The hydration media became quite cloudy and empty pockets were observed in the films containing the suspension. Similarly a small percentage of microcapsules were lost from the films during manipulation.

In a separate study the hydration properties of PVA 425 hydrogels containing tetracycline free base were evaluated. Hydrogels prepared from PVA 523 could not be evaluated, since the matrix was not stable and was significantly solubilized. These materials require reinforcement, such as the foam backing of the wound dressings to maintain integrity. As shown in Table 9 the increase in diameter of the tetrayclcine loaded PVA 425 was very similar to that previously observed for chlorhexidine diphosphanilate in this matrix (Table 10). Weight increased from than 6 fold in 45 minutes. The smaller weight increase observed for chlorhexidine loaded PVA 425 is probably due to loss of drug suspension during the 24 hour hydration period used in the earlier study. Only one matrix prepared from PVA blends, 53% PVA 425 and 27% PVA 523, could be studied. At higher PVA 523 concentrations the gels were too soluble for accurate measurement. The increase in diameter of the drug loaded PVA blend matrix (Table 9) was somewhat less than observed for PVA 425 and the increase in weight was significantly less.

Similar studies were conducted with poly-HEMA hydrogels. There were no differences in the water uptake of the different hydrogels (Table 10). Based on the dehydrated weight all samples increased by approximately 160%. Film thickness was slightly dependent upon initial HEMA concentration during polymerization. Materials prepared with either 30 or 40% water were thicker following hydration than samples prepared with 20% water.

3. Antimicrobial Efficacy

a. Sensitivity

Sensitivity tests were conducted as described (Section III.A.4). The streptococci were more sensitive to the antimicrobials than the staphylococci, and chlorhexidine diphosphanilate was more potent that tetracycline (Tables 11 and 12). In the first sensitivity test degradation of tetracycline did occur. In the second test all strains except S. aureus 29996 showed an order or magnitude increase in sensitivity. Based on the results of both sensitivity studies the minimum inhibitory concentration of chlorhexidine diphosphanilate is 0.41 μg/ml for the streptococci and 4 μg/ml for staphylococci. To insure a therapeutic response the sustained release matrix should maintain a concentration of 4 μg/ml for strep. infections and 40 μg/ml for staph. Similarly the requirements for insuring a tetracycline therapeutic response are 5 μg/ml for strep. and 60 μg/ml for staph. As shown in Section III.B.1 with PVA 425 matrixes under sink conditions, tetracycline levels exceeded 9 μg/ml for 9 days and 25 μg/ml for 2 days and chlorhexidine diphosphanilate concentration exceeded 16 μg/ml for 2 days. Clearly under in vivo conditions therapeutic levels can be anticipated especially for streptococci.

Disc sensitivity assay was conducted on the two materials selected for in vivo evaluation, PVA-425 matrix plasticized with 20% glycerol containing 20% chlorhexidine diphosphanilate, 20% tetracycline free base, or no drug for con-

trol. As shown in Table 13 significant inhibition of bacterial growth was seen for all strains and both formulations. On control plates of S. pyogenes 2 of 4 control discs had no bacterial growth under the film but inhibition did not extend beyond the margin of the disc. This may indicate some antimicrobial activity of the matrix itself. The area of inhibition was much greater with tetracycline than with chlorhexidine diphosphanilate and there was little difference observed between strains. The difference between drugs probably is a reflection of the solubility difference between the drugs. When chlorhexidine diphosphanilate discs were removed from the agar surface a white precipitate, probably the drug, was left behind. The tetracycline discs were much darker after they were placed on the agar indicating that the drug had decomposed during the test. In order to further evaluate the antimicrobial efficacy of these discs, they were removed from the first plate and placed on a second seeded plate. The zone of inhibition in this 48 hour test was not significantly different than on the first test. This indicates that diffusion of active antimicrobial agents was quite similar over a 24-48 hour period.

b. Efficacy

The reproducibility of the assay system for determination of the number of viable bacteria was studied. Viable bacteria are defined as colony forming units (cfu). In one study two dilutions of the stock cultures (10^3 and 10^4) were plated out in quadruplicate. As shown in Table 14 the relative standard errors of the mean were less than 5% which indicates good reproducibility in plating out a dilution from the stock cultures. When multiple dilutions from the stock cultures were plated out (a single plate for each dilution) reproducibility was not as good. However, the relative standard errors of the mean were still less than 10% (Table 15).

In order to determine efficacy the number of bacteria remaining on the wound site must be determined. The method is based on that described by Setterstrom, et al., 1985. Since a detergent, Triton X-100, is utilized in the recovery buffer and diluent, the effect of the detergent on S. pyogenes 14289

viability was determined. On the day following the reproducibility study dilutions were prepared from the stock culture in 40 mM, pH 7.4 phosphate buffered saline containing different concentrations of Triton X-100 and plated. As shown in Table 15, there is a slight, 9%, but insignificant decrease in observed cfu when the detergent at 0.1% (v/v) is compared to the control. Comparison of the cfu/ml in Tables 14 and 15 for S. pyogenes 14289 shows that the count of viable bacteria decreased significantly when the cultures were maintained for 48 rather than 24 hours. Note that in this study where n = 4 for each group the relative standard errors of the mean ranged from 7 to 11%. This compares well with the reproducibility study.

A pilot study was conducted to determine if an infection could be established in a guinea pig wound model. Six guinea pigs were prepared as described, and 100μl of one of the two strains of streptococcus pyogenes was pipetted into each wound. Guinea pigs were housed separately for three days. On the third day, they were euthanized, dressings were removed, and two 1 ml washes of sterile saline were used to recover bacteria from the wound. These extracts were then serially diluted and plated out in duplicate. Colony forming units per cm^2 were calculated to be from 1.3×10^7 to 3.5×10^8. In three wounds initially infected with 2.4×10^6 cfu of S. pyogenes 14289, a mean of 1.38×10^8 cfu/cm^2 were recovered on day 3. In 3 wounds initially infected with 3.0×10^6 cfu S. pyogenes 19615, a mean of 4.1×10^7 cfu/cm^2 were recovered. Despite the degree of variability it is evident that an infection can be established in a wound in three days. The 100 μl innoculum was difficult to localize in the wound, and this was reduced to 50 μl in subsequent studies.

The PVA 425, 20% glycerol plasticized matrix was selected for the initial in vivo efficacy studies since it is more stable than other PVA matrixes and provided sustained release of both antimicrobial agents. Based on these pilot studies 3 groups of 5 guinea pigs were infected with 50 μl of a 24 hour stock culture of S. pyogenes 14289 containing 5.59×10^8 cfu/ml ($\pm$ 1.93×10^7

S.E.M., n = 5). On day 3 they were treated with PVA-425 - 20% glycerol matrix containing 20% chlorhexidine diphosphanilate, 20% tetracycline free base or no drug for controls. Wounds were evaluated on day 6. As shown in Table 1, both wound dressings gave very significant reductions in bacterial counts. With chlorhexidine diphosphanilate 4 to 5 wounds were sterile and only 1 plate of the 20 duplicates showed any growth at all. Tetracycline was less effective. Only 40% of the plates showed no growth, and 1 to 8 colonies were observed on the others. There was 100% infection rate for the controls, although the bacterial concentration was nearly 2 orders of magnitude lower than was observed in the pilot study 3 days after infection.

The hydrogels removed from the wounds were soft, pliable, and intact. Chlorhexidine diphosphanilate gels had clear areas, apparently free of drug, and opaque areas apparently containing white drug precipitate. Tetracycline gels had larger clear areas, and the drug remaining in the gel appeared to be a greenish brown. This again may indicate degradation. Control wounds had significant amounts of purulent exudate while treated wounds were drier with no evidence of purulence. While all wounds showed some evidence of healing at day 6, the control wounds resembled the wounds on day 3 and were more inflammed than drug treated wounds.

Efficacy was clearly demonstrated against an established Streptococcus pyogenes 14289 infection. Therefore efficacy was tested against two additional models Staphylococcus aureus strain 29247 and a mixed infection initiated with 1.00×10^7 cfu streptococci and 6.8×10^6 cfu staphylococcus. The established infections were treated on day 3 and bacterial content of the wounds was evaluated after 6 days of treatment. As shown in Table 2 treatment of S. aureus or mixed bacterial infection with the 20% chlorhexidine diphosphanilate wound dressing gave very significant improvement. There were, however, no sterile wounds sicne at least 1 of the quadruplicate plates for each wound had 32 of more cfu/cm^2. The wound and dressings removed from the wounds appeared the same as in the previous study.

IV. CONCLUSIONS

PVA based hydrogels were investigated most intensely during the 6 month Phase I feasibility study to provide a model for continued research and development of BIOTEK's antimicrobial wound dressings. Rate of drug release from PVA matrixes was dependent upon drug concentration and solubility of drug. Release rates were greatest with the more soluble drug and at higher drug concentration. Release rates were clearly dependent upon physical properties of the hydrogel matrix. The less water soluble fully hydrolyzed PVA's provided more sustained release and maintained their integrity for longer periods. Increasing the degree of crosslinking significantly decreased the rate of drug release. Release was also dependent upon the concentration of plasticizer.

Similar studies were conducted with wound dressings based on poly-HEMA hydrogels. Release rates of antimicrobial agents increased with increasing water content of the gels. As was observed with PVA based hydrogels increasing the crosslinking density decreased the rate of release. In vitro release studies were also conducted with hydrogel matrixes of polyethylene oxide and polyacrylamide.

In vitro sensitivity tests demonstrated that all four tested strains, S. aureus 29247 and 29996 and S. pyogenes 14289 and 19615, were sensitive to tetracycline and chlorhexidine diphosphanilate. S. pyogenes strains were more sensitive than S. aureus, and chlorhexidine diphosphanilate was more potent than tetracycline. Sensitivity was also demonstrated for drug loaded hydrogels. Significant inhibition of bacterial growth on agar plates was observed for PVA matrixes containing either agent. Tetracycline release was approximately constant for 48 hours and effectively inhibited bacterial growth, although there was evidence of tetracycline degradation in the matrix. Based on the sensitivity and in vitro release studies, tetracycline release under sink conditions can maintain a tetracycline concentration 10 times higher than the minimum inhibitory concentration for at least a week.

In vivo efficacy was evaluated in a model based on that of Setterstrom, *et al.* (1985) with the microbial quantitation system of Williamson and Klingman (1965). Both chlorhexidine diphosphanilate and tetracycline in PVA based wound dressings were efficacious against established infections. Chlorhexidine diphosphanilate provided a larger decrease in microbial counts than tetracycline and provided a sterile site for wound healing.

The major objectives of this SBIR Phase I feasibility study have been achieved. The methods necessary for further research on antimicrobial wound dressing have been developed and tested. The results of the experiments conducted clearly demonstrate that an efficacious, antimicrobial wound dressing based on hydrogels can be developed within the framework of the SBIR program. These preliminary studies will provide the basis for a Phase II SBIR proposal.

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TABLE 1

IN VIVO EFFICACY*

S. Pyogenes 14289
PVA 425 - 20% Glycerol Matrix

Test Material	Control	20% Chlorhexidine Diphosphanilate	20% Tetracycline Base
cfu/cm^2 wound	2.46×10^6	15.8**	395**
S.E.M.	4.3×10^5	15.8	160
n	5	5	5

---

* Wounds were initially infected with 50 μl of 5.59×10^8 cfm/ml culture = 2.79×10^7 cfu total. Wounds were treated 3 days after infection and assayed 6 days following treatment.

** $p < 0.001$

TABLE 2

IN VIVO EFFICACY*

PVA 425 - 20% Glycerol Matrix 20% Chlorhexidine Diphosphanilate

	Control	Treated	
	S. aureus	S. aureus	Mixed Infection
mean cfu/cm² wound	7.10×10^6	304**	13.2**
S.E.M.	1.06×10^5	81.3	6.2
n	5	4***	5

---

* Wounds were initially infected with 50 μl of 2.70×10^8 cfu of S. aureau 29247 culture = 1.35×10^7 cfu total, or 25 μl of the S. aureus culture plus 25 μl of 4.3×10^8 cfu/ml of s. pyogenes 14289 culture = 1.75×10^7 total cfu for a mixed infection. Wounds were treated 3 days after infection with 20% chlorhexidine diphosphanilate in PVA 425 - 20% glycerol matrix, and assayed 6 days following treatment.

** $p < 0.001$

*** One wound was contaiminated

TABLE 3

CHARACTERISTICS OF POLYVINYL ALCOHOLS

	PVA 350	PVA 425	PVA 523
Percent Hydrolysis	98.0-98.8	95.5-96.5	87.0-89.0
Viscosity*	55-65	26-30	22-26
Molecular Weight	106,000-110,000	77,000-79,000	77,000-79,000
pH**	5.0-7.0	4.5-6.5	4.0-6.0

*CPS of 4% aqueous solution, 20°C

**4% aqueous solution

TABLE 4

CHLORHEXIDINE DIPHOSPHANILATE MICROCAPSULES

Size Range μm	Recovery %
<105	0.3
105-210	5.7
210-300	10.0
300-345	25.8
425-600	26.8
>600	31.3

Drug Concentration = 69%

TABLE 5

TETRACYCLINE HYDROCHLORIDE

CELLULOSE TRIACETATE MICROCAPSULES*

Size Range (um)	Recovery %
<106	2.5
106-212	37.1
212-300	22.0
300-425	26.4
425-600	11.4
>600	0

*Total Yield = 87.6%
Nominal Drug Concentration = 80%

TABLE 6

POLYETHYLENE OXIDE HYDROGELS

Tetracycline Release Characteristics

Sample	Time Interval[1] (Hr)	Rate[2] (ug/hr)	24 Hour Cumulative Release (mg)
Soaked Gel	24-42	136.4	52.6
Hydrochloride	10-42	72.8	9.69
Free Base	6-205	47.5	1.47
Microcapsules			
106-212 μm	10-24*	49.3	2.78
212-300 μm	10-24*	58.5	5.01
300-425 μm	20-42*	45.3	7.61
426-600 μm	20-42*	49.1	8.20

[1] Time interval during which the rate of drug release was linear with a least squares correlation coefficient > 0.98.

[2] Rate of drug release during the linear phase of release.

* Microcapsule release was followed for either 24 or 42 hours.

TABLE 7

SWELLING OF CHLORHEXIDINE DIPHOSPHANILATE HYDROGELS*

	Diameter**				Thickness**	
	45 Minutes		27 Hours		45 Minutes	
	Water	Saline	Water	Saline	Water	Saline
Control	164	164	187	185	164	161
23% Chlorhexidine Suspension	171	---	180	179	173	---
20% Chlorhexidine Microcapsules	140	140	163	156	110	112
20% Chlorhexidine Microcapsules 1.5% Glutaraldehyde	180	140	178	174	120	129
23% Chlorhexidine Suspension 1.5% Glutaraldehyde	146	146	150	165	171	165

* Films were prepared with a base of PVA 425 with 20% glycerol (w/w)

** Data are expressed as percent of initial values. Initial diameter = 0.25 inches. Mean initial thickness = 0.41 for microcapsule coating films and 0.20 for others.

TABLE 8

HYDRATION OF CHLORHEXIDINE DIPHOSPHANILATE PVA 425 HYDROGELS*

Sample	Distilled Water	Saline 0.9%
Control	211	211
23% Chlorhexidine Suspension	192	225
20% Chlorhexidine Microcapsules	196	250
20% Chlorhexidine Microcapsules 1.8% Glutaraldehyde	139	175
23% Chlorhexidine Suspension 1.8% Glutaraldehyde	161	171

* Films were prepared with a base of PVA 425 with 20% glyercol as a plasticizer. Data are expressed as percent of dry weight following hydration for 24 hours.

TABLE 9

HYDRATION OF PVA HYDROGELS*

Sample	Diameter**			Weight**		
	5 min.	30 min.	45 min.	15 min.	30 min.	45 min.
20% Tetracycline Base - PVA 425	128	157	171	456	650	621
20% Chlorhexidine Diphosphanilate - PVA Blend***	114	143	157	262	392	350

* Discs were cut from dry films, measured and placed in 2 ml of distilled water. At 15, 30, and 45 minutes following immersion, samples were blotted and remeasured.

** Data is expressed as percent of initial dry diameter or weight.

*** The matrix was composed of 53% PVA 425, 77% PVA 523, and 20% glycerol.

TABLE 10

HYDRATION OF HEMA HYDROGELS*

Sample	Thickness**	Weight**
80% HEMA 0.5% EGDMA	106	163
80% HEMA 1.0% EGDMA	106	160
70% HEMA 0.5% EGDMA	112	166
70% HEMA 1.0% EGDMA	120	164
60% HEMA 1.0% EGDMA	121	165

* Discs were cut from hydrated hydrogels and dried at 100°C for 24 hours. Distortion during drying prevented measurement of diameters. Thickness and weight of the samples were determined and samples placed in distilled water. Samples were blotted and measured 60 minutes after immersion.

** Data are presented as percent of dehydrated values.

TABLE 11

ANTIMICROBIAL SENSITIVITY*

Chlorhexidine Diphosphanilate

Strain/Concentration (ug/ml)	619	52.5	4.45	0.377	0.032
S. aureus 29996	-	-	-	+	+
S. aureus 29247	-	-	-	+	+
S. pyogenes 19615	-	-	-	-	+
S. pyogenes 14289	-	-	-	-	+

Tetracycline Hydrochloride**

Strain/Concentration (ug/ml)	856	72.5	6.15	0.521	0.0441
S. aureus 29996	-	-	+	+	+
S. aureus 29247	-	-	+	+	+
S. pyogenes 19615	-	-	-	+	+
S. pyogenes 14289	-	-	-	+	+

---

* + Growth present

  - Growth absent.

** Sterilized in solution at indicated concentration

TABLE 12

ANTIMICROBIAL SENSITIVITY*
TETRACYCLINE FREE BASE**

Strain/concentration (μg/ml)	6.10	0.517	0.044
S. aureus 29996	+	+	+
S. aureus 29247	-	+	+
S. pyogenes 19615	-	-	+
S. pyogenes 14289	-	-	+

---

* + Growth present
  - Growth absent

** Sterilized in dry nitrogen atmosphere

TABLE 13

DISC SENSITIVITY ASSAY

Strain	S. p. 14289	S. p. 19615	S.a. 29247	S.a. 29996
20% chlorhexidine 24 hour	13.0* ±0.4 n=4	16.0 n=2	15.0 n=2	16.0 n=2
20% tetracycline 24 hour	25.8 ±0.9 n=4	25.0 n=2	24.5 n=2	26.0 n=2
20% tetracycline 24 hour	23.2 ±0.9 n=4	--	--	--

7 mm discs of 425 PVA-20% glycerol with drug were placed on Standard Methods Agar plates seeded with the strain noted. Following incubation for 24 hours, diameters of inhibition zones were measured. Discs were 9 mm in diameter at end of study. In the S. pyogenes study 2 of 4 control discs had no bacterial growth under the film, but no control showed inhibition greater than diameter of the disc.

* Data are presented as mean diameter (mm) of inhibition ± S.E.M.

** Following initial 24 hour test, discs were placed on freshly streaked plates.

TABLE 14

REPRODUCIBILITY OF BACTERIAL COUNTS*

	S. Pyogenes 14289	S. Pyogenes 19615
Single dilution/ Multiple plates	$2.34 \times 10^8 \pm 1.06 \times 10^7$ n = 8	$1.81 \times 10^8 \pm 7.38 \times 10^6$ n = 7**
Multiple dilutions/ Single Plates	$2.46 \times 10^8 \pm 2.23 \times 10^7$ n = 7**	$3.11 \times 10^8 \pm 3.06 \times 10^7$ n = 8

*Data are presented as mean cfu/ml of stock cultures ± S.E.M. Stock cultures were diluted in 40 mM pH 7.4 phosphate buffered saline and pour plates of appropriate dilutions, 10^3 and 10^4, were prepared and counted.

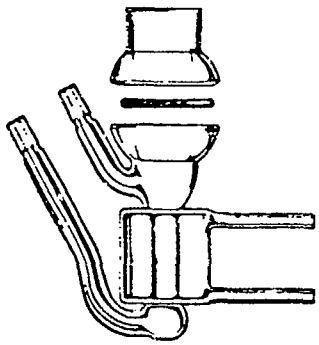
**One plate was discarded since the cell count was 2.6 to 6.6 times greater than the mean.

TABLE 15

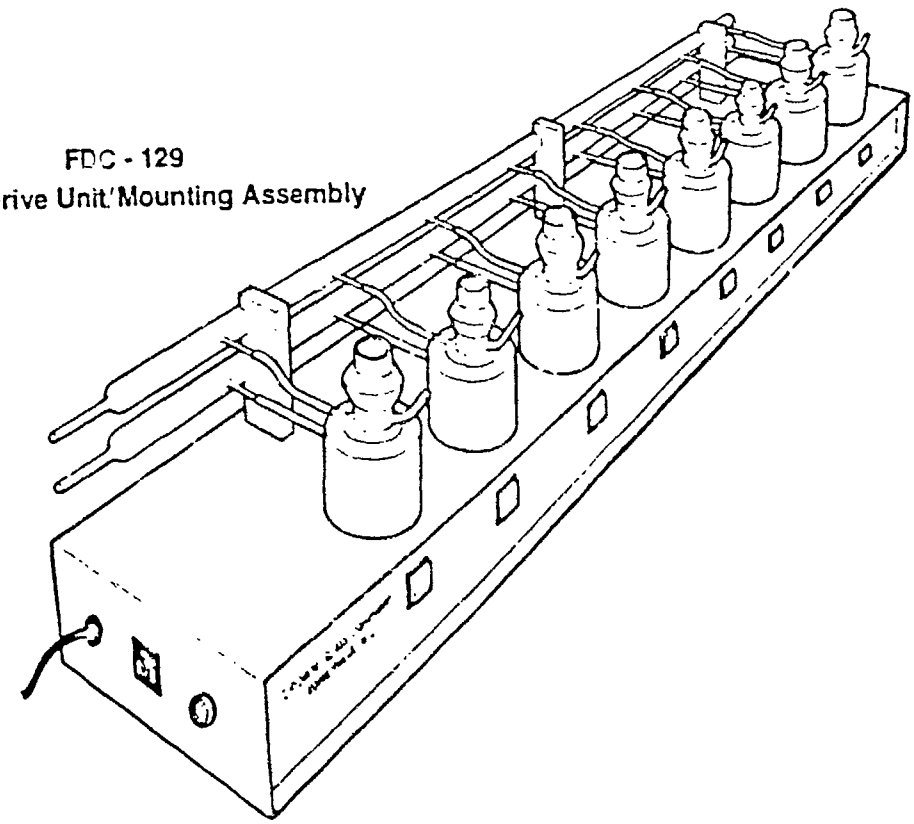
EFFECT OF DETERGENT ON S. PYOGENES VIABILITY

Triton X-100 % (v/v)	cfu/ml* ± S.E.M.
0	8.38×10^7 $\pm 9.40 \times 10^6$
.001	9.38×10^7 $\pm 6.25 \times 10^6$
.01	7.92×10^7 $\pm 8.32 \times 10^6$
0.1	7.62×10^7 $\pm 6.30 \times 10^6$

*Data are presented as mean cfu/ml of stock culture ± S.E.M.
n = 4 for each group



FDC-200
Flow-Through Franz Diffusion Cell



FDC - 129
8-cell Drive Unit/Mounting Assembly

FIGURE 1

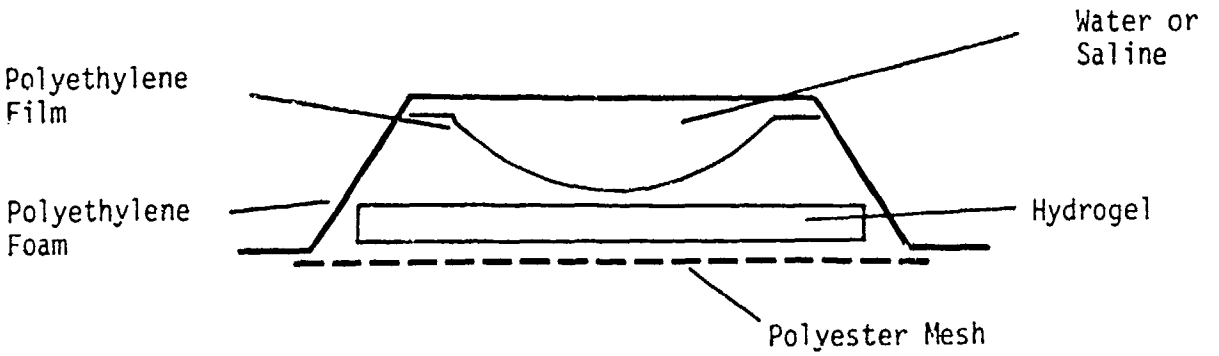


FIGURE 2 Hydrogel Support

FIGURE 3

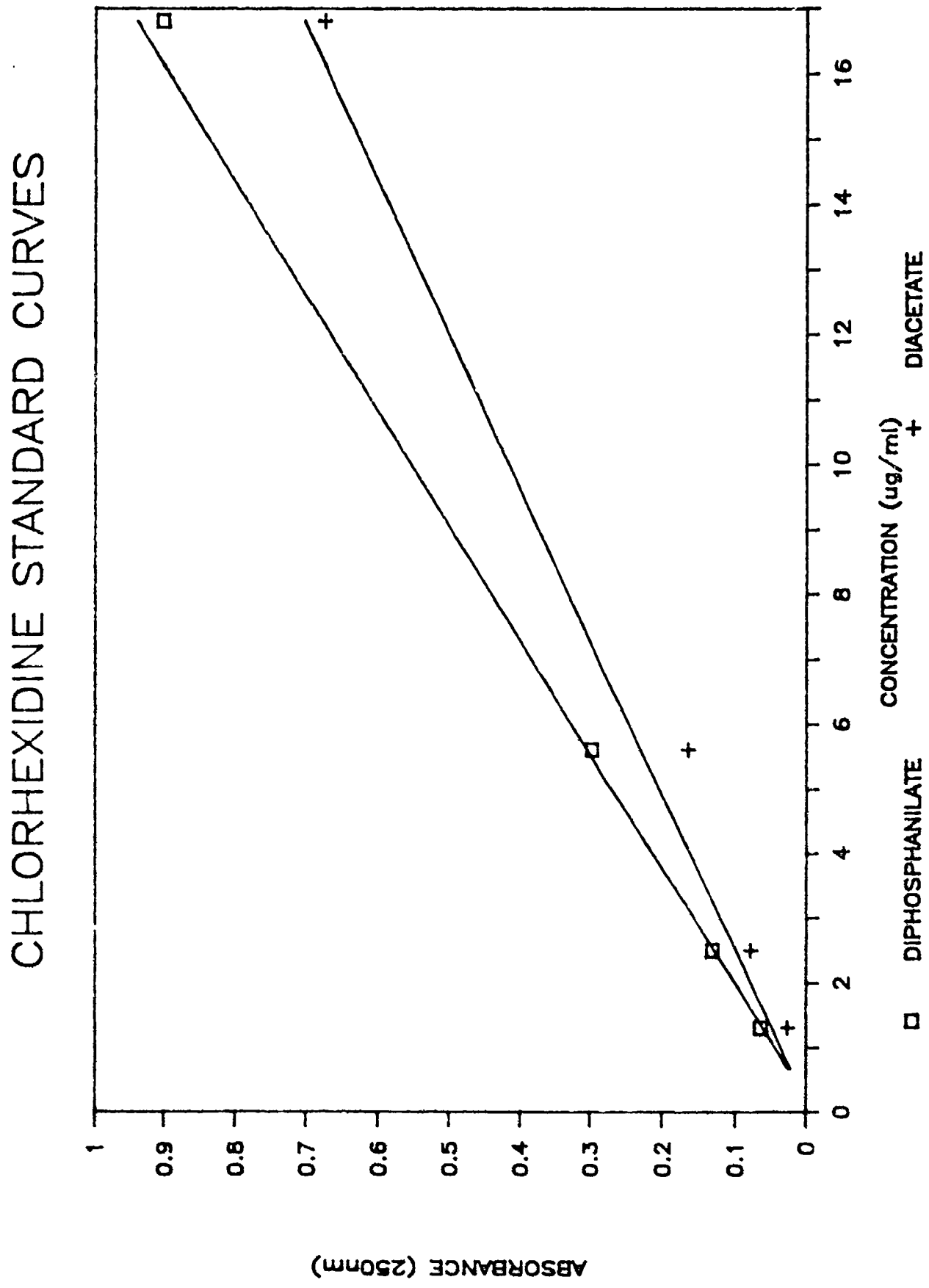

CHLORHEXIDINE STANDARD CURVES
ABSORBANCE (250nm)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14
16
CONCENTRATION (ug/ml)
DIPHOSPHANILATE
DIACETATE

FIGURE 4

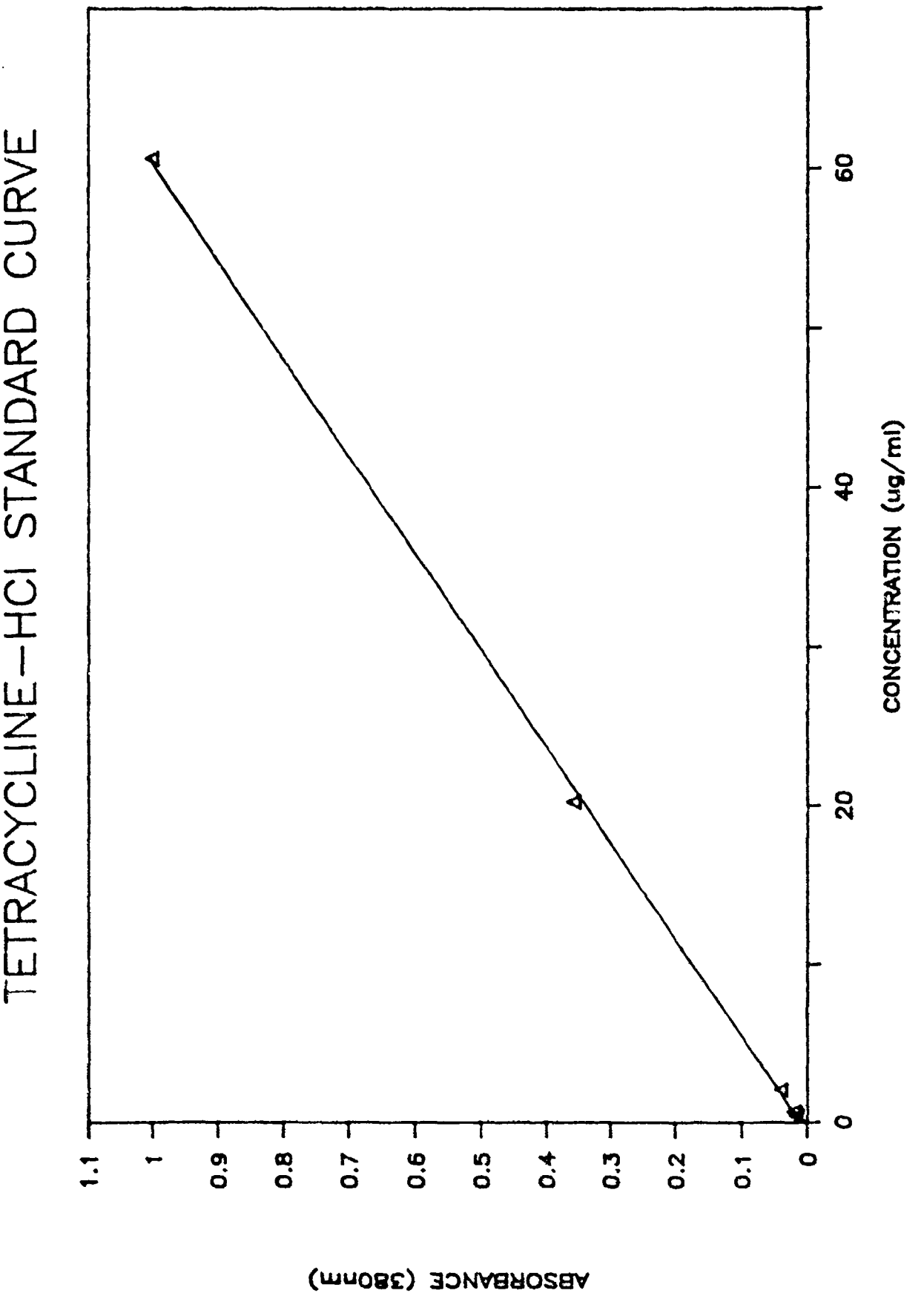

TETRACYCLINE—HCl STANDARD CURVE
ABSORBANCE (380nm)
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
CONCENTRATION (ug/ml)

FIGURE 5

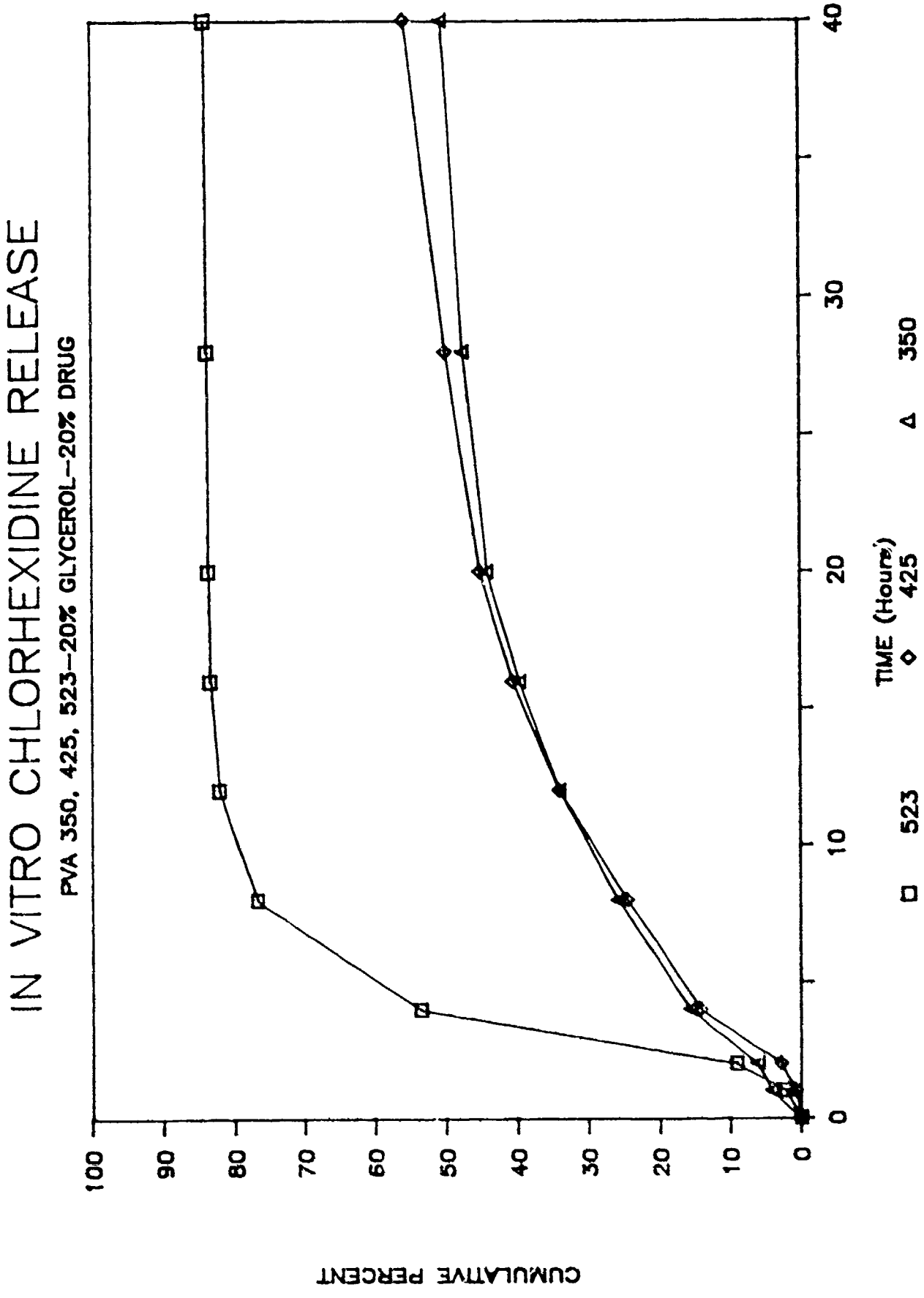

IN VITRO CHLORHEXIDINE RELEASE
PVA 350, 425, 523–20% GLYCEROL–20% DRUG
CUMULATIVE PERCENT
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
TIME (Hours)
□ 523
◇ 425
△ 350

FIGURE 6

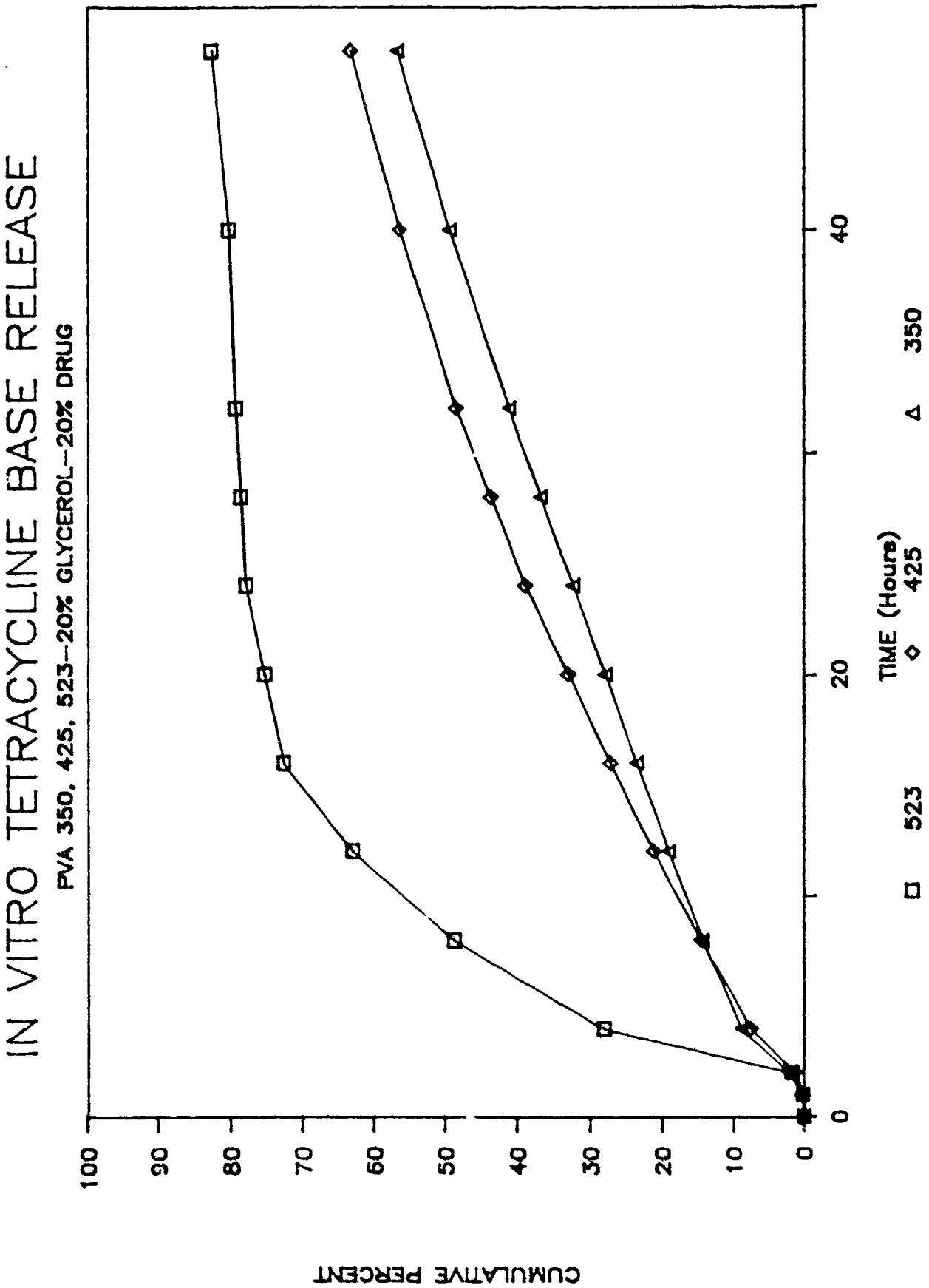

IN VITRO TETRACYCLINE BASE RELEASE
PVA 350, 425, 523–20% GLYCEROL–20% DRUG
CUMULATIVE PERCENT
100
90
80
70
60
50
40
30
20
10
0
0
20
40
TIME (Hours)
□ 523
◇ 425
△ 350

FIGURE 7

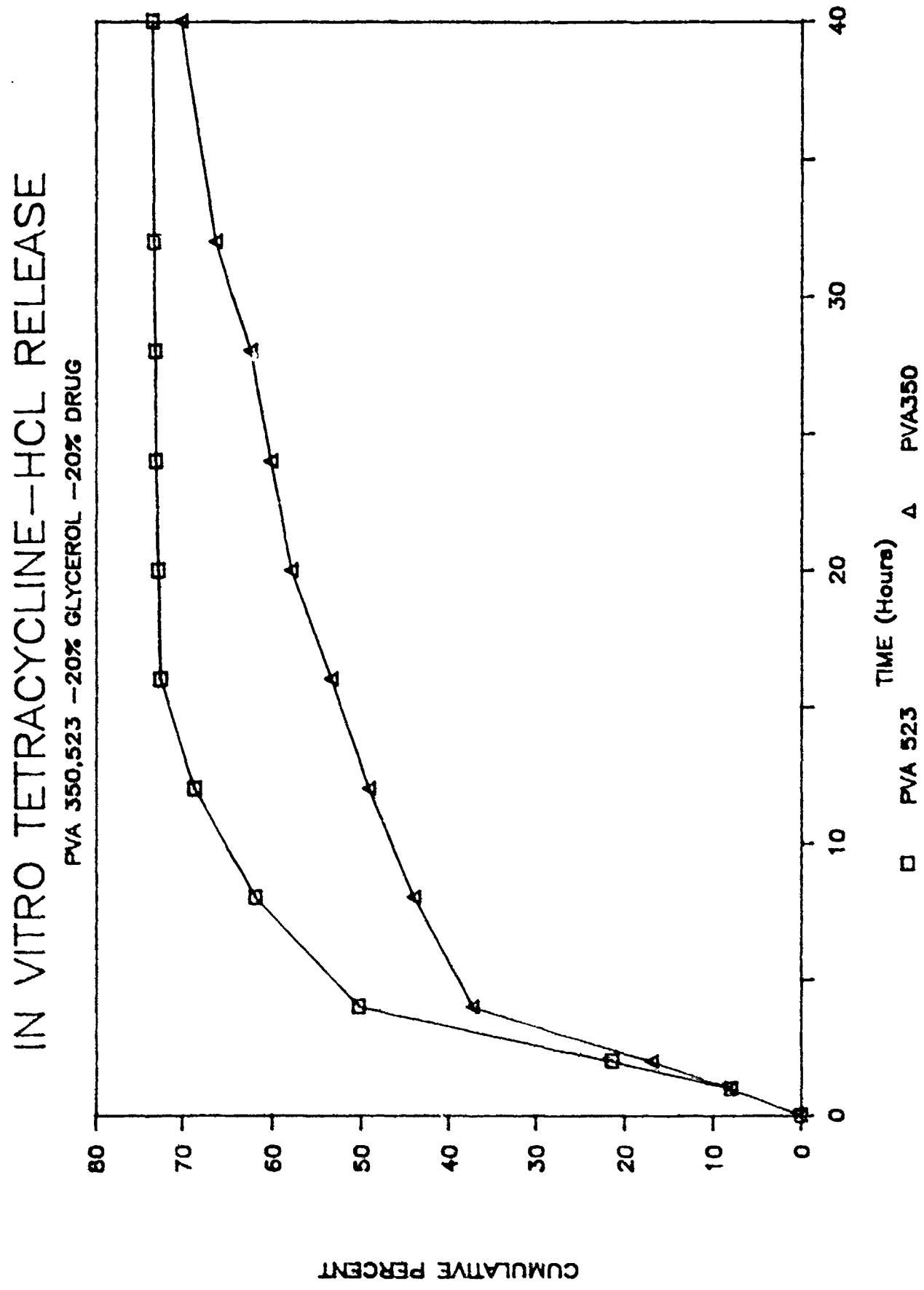

IN VITRO TETRACYCLINE–HCL RELEASE
PVA 350,523 –20% GLYCEROL –20% DRUG
CUMULATIVE PERCENT
0
10
20
30
40
50
60
70
80
0
10
20
30
40
TIME (Hours)
□ PVA 523
△ PVA350

FIGURE 8

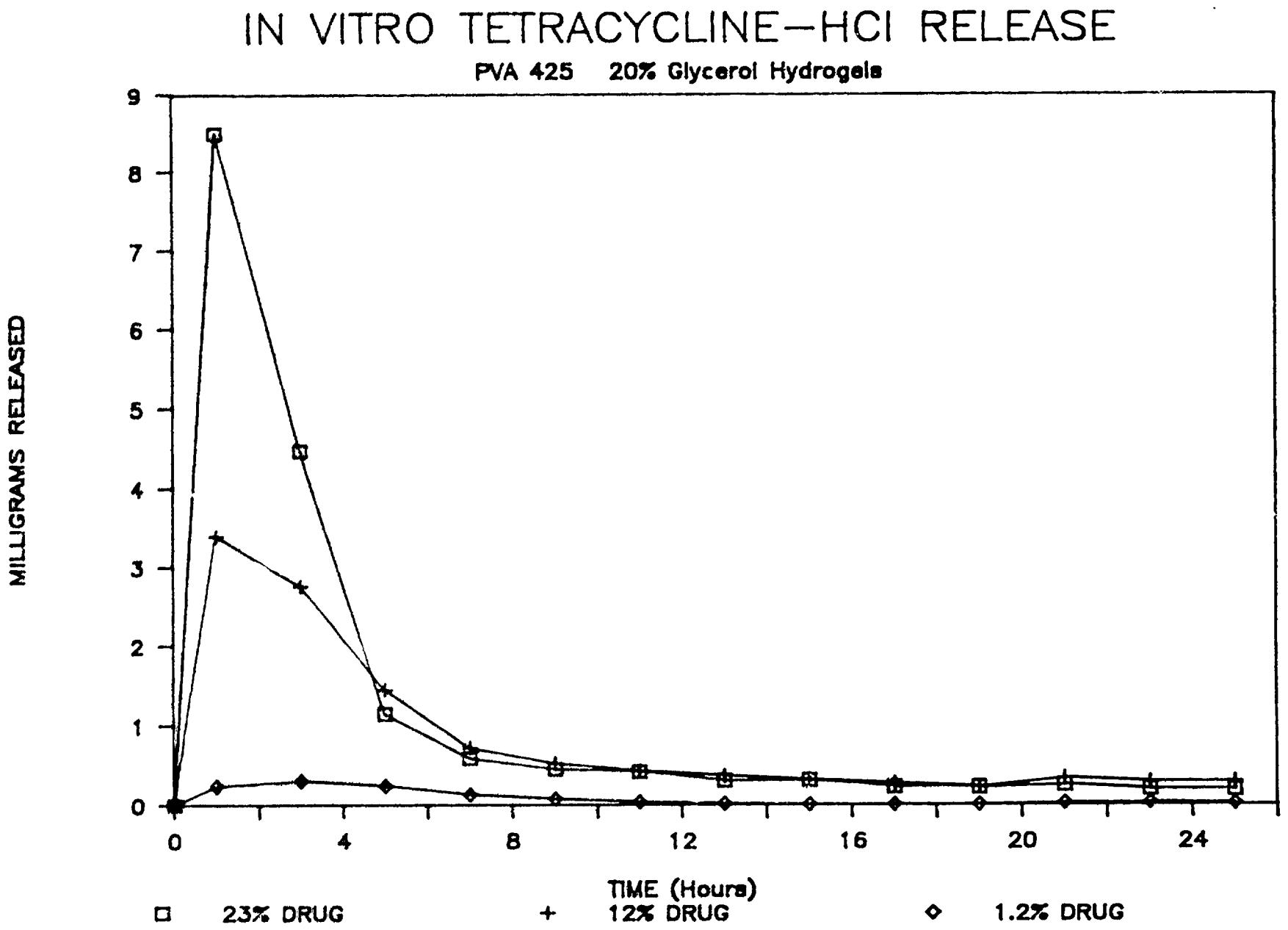

IN VITRO TETRACYCLINE–HCl RELEASE
PVA 425 20% Glycerol Hydrogels
MILLIGRAMS RELEASED
0
1
2
3
4
5
6
7
8
9
0
4
8
12
16
20
24
TIME (Hours)
23% DRUG
12% DRUG
1.2% DRUG

FIGURE 9

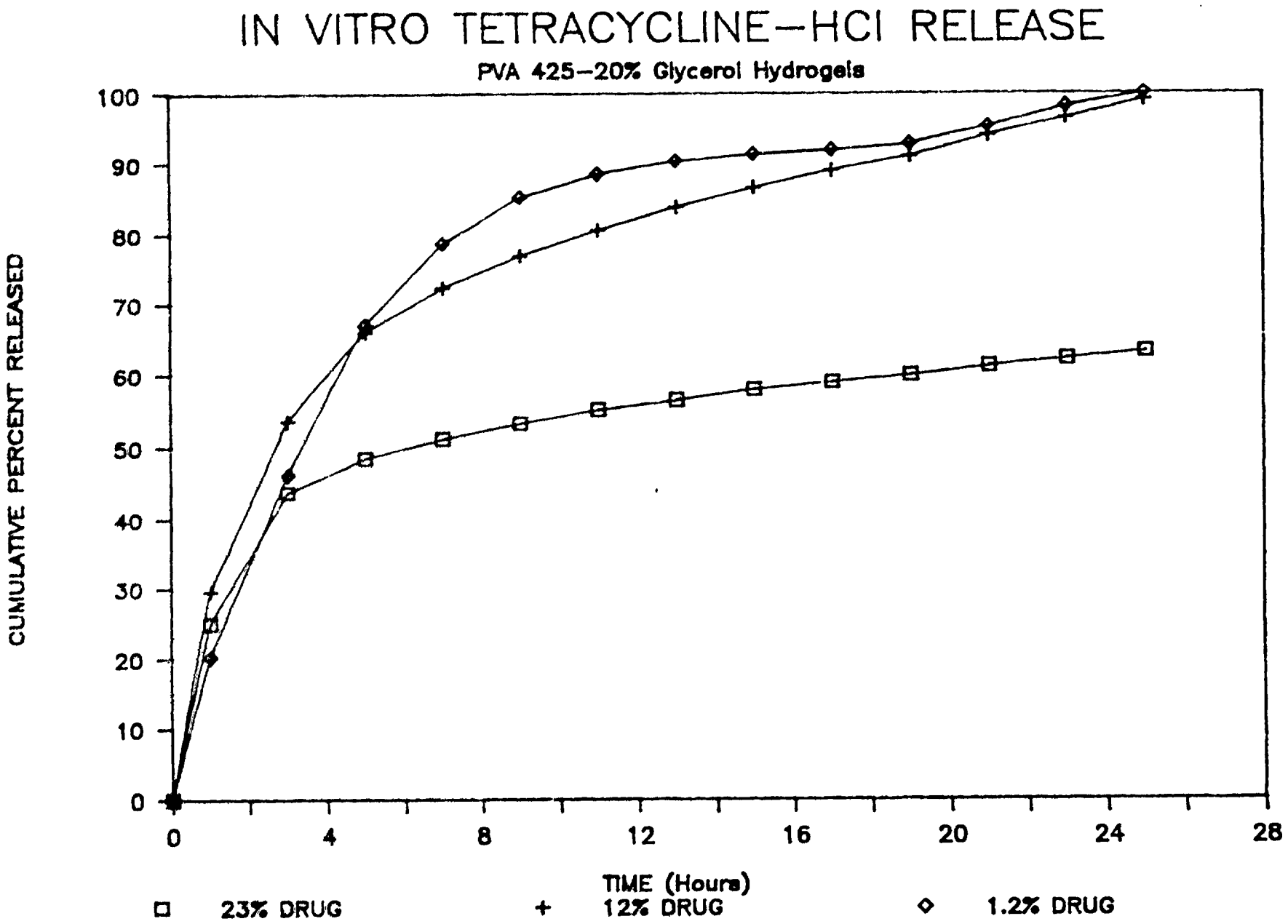

IN VITRO TETRACYCLINE–HCl RELEASE
PVA 425–20% Glycerol Hydrogels
CUMULATIVE PERCENT RELEASED
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
28
TIME (Hours)
23% DRUG
12% DRUG
1.2% DRUG

FIGURE 10

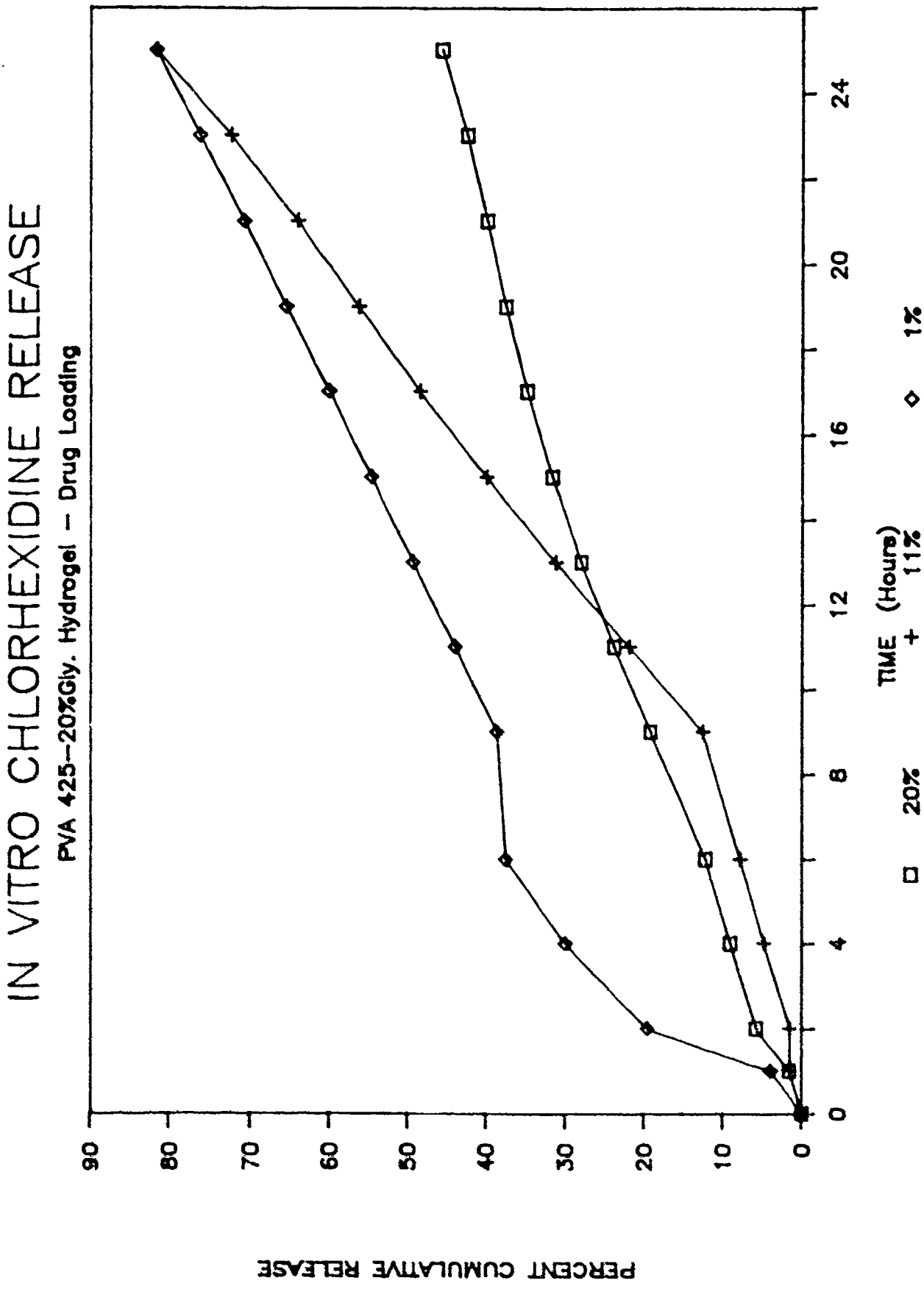

IN VITRO CHLORHEXIDINE RELEASE
PVA 425--20%Gly. Hydrogel -- Drug Loading
PERCENT CUMULATIVE RELEASE
0
10
20
30
40
50
60
70
80
90
0
4
8
12
16
20
24
TIME (Hours)
□ 20%
+ 11%
◇ 1%

FIGURE 11

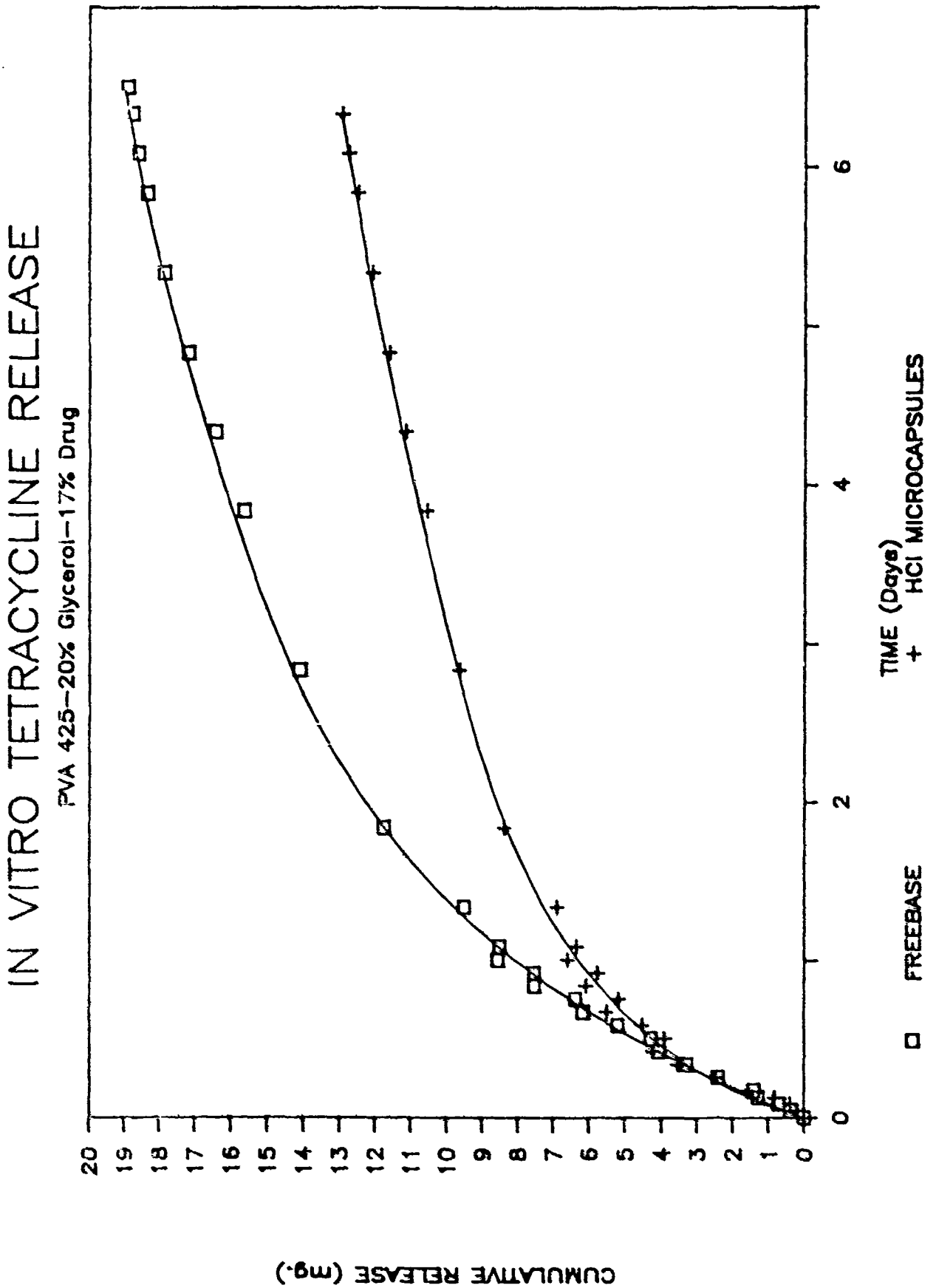

IN VITRO TETRACYCLINE RELEASE
PVA 425–20% Glycerol–17% Drug
CUMULATIVE RELEASE (mg.)
TIME (Days)
□ FREEBASE
+ HCl MICROCAPSULES
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

FIGURE 12

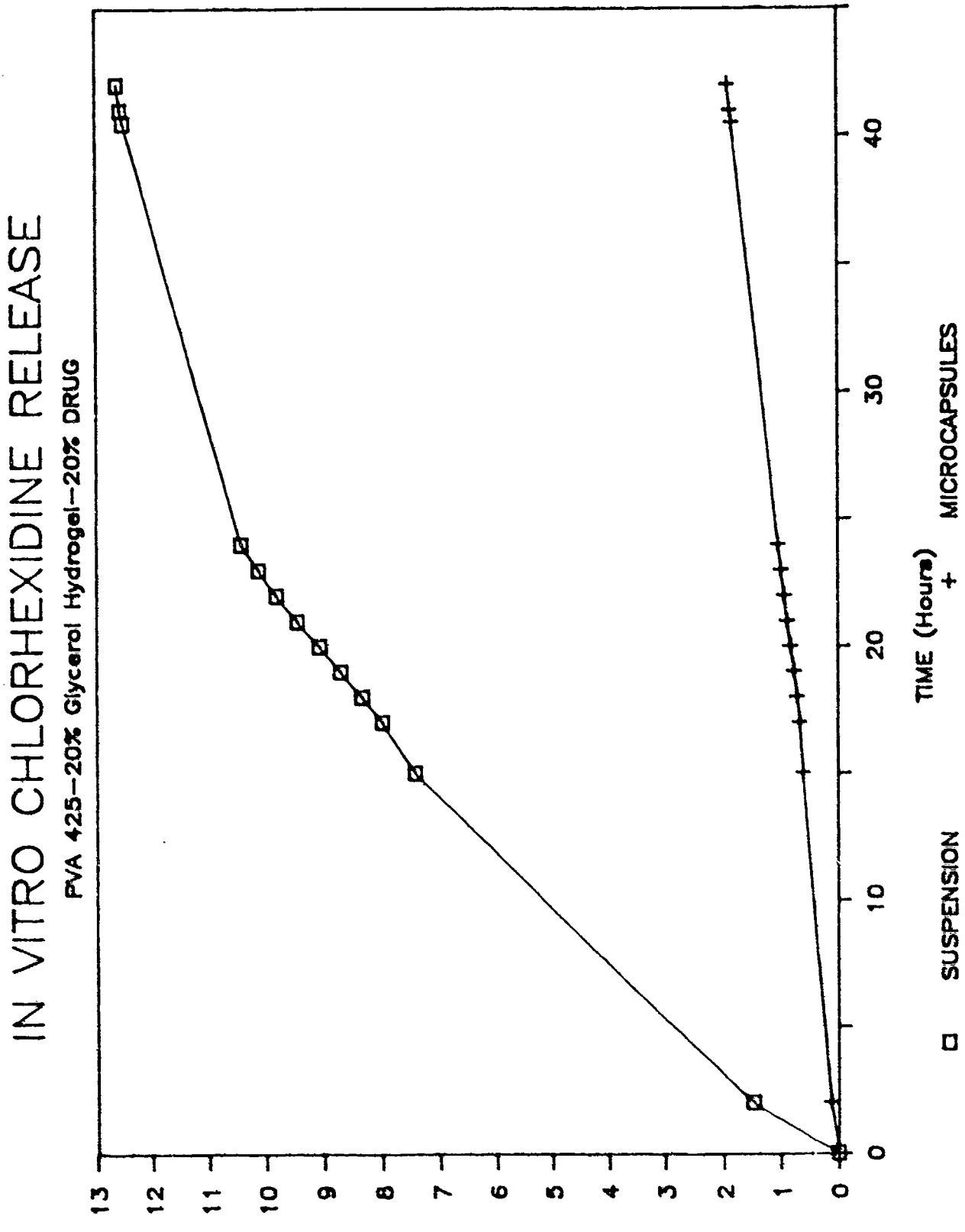

IN VITRO CHLORHEXIDINE RELEASE
PVA 425—20% Glycerol Hydrogel—20% DRUG
CUMULATIVE RELEASE (mg.)
TIME (Hours)
□ SUSPENSION
+ MICROCAPSULES
0
1
2
3
4
5
6
7
8
9
10
11
12
13
0
10
20
30
40

FIGURE 13

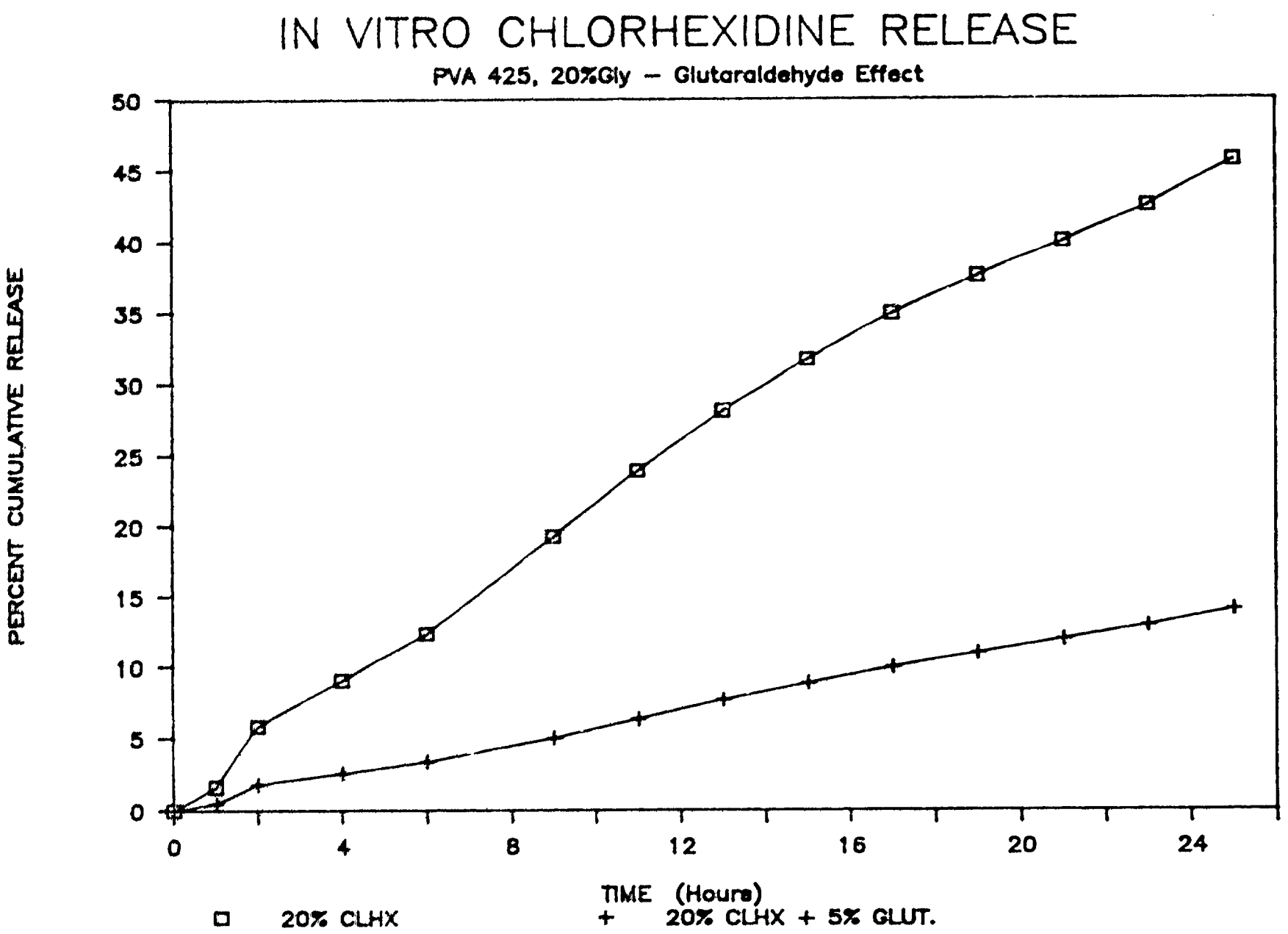

IN VITRO CHLORHEXIDINE RELEASE
PVA 425, 20%Gly – Glutaraldehyde Effect
PERCENT CUMULATIVE RELEASE
50
45
40
35
30
25
20
15
10
5
0
0
4
8
12
16
20
24
TIME (Hours)
20% CLHX
20% CLHX + 5% GLUT.

FIGURE 14

IN VITRO CHLORHEXIDINE RELEASE

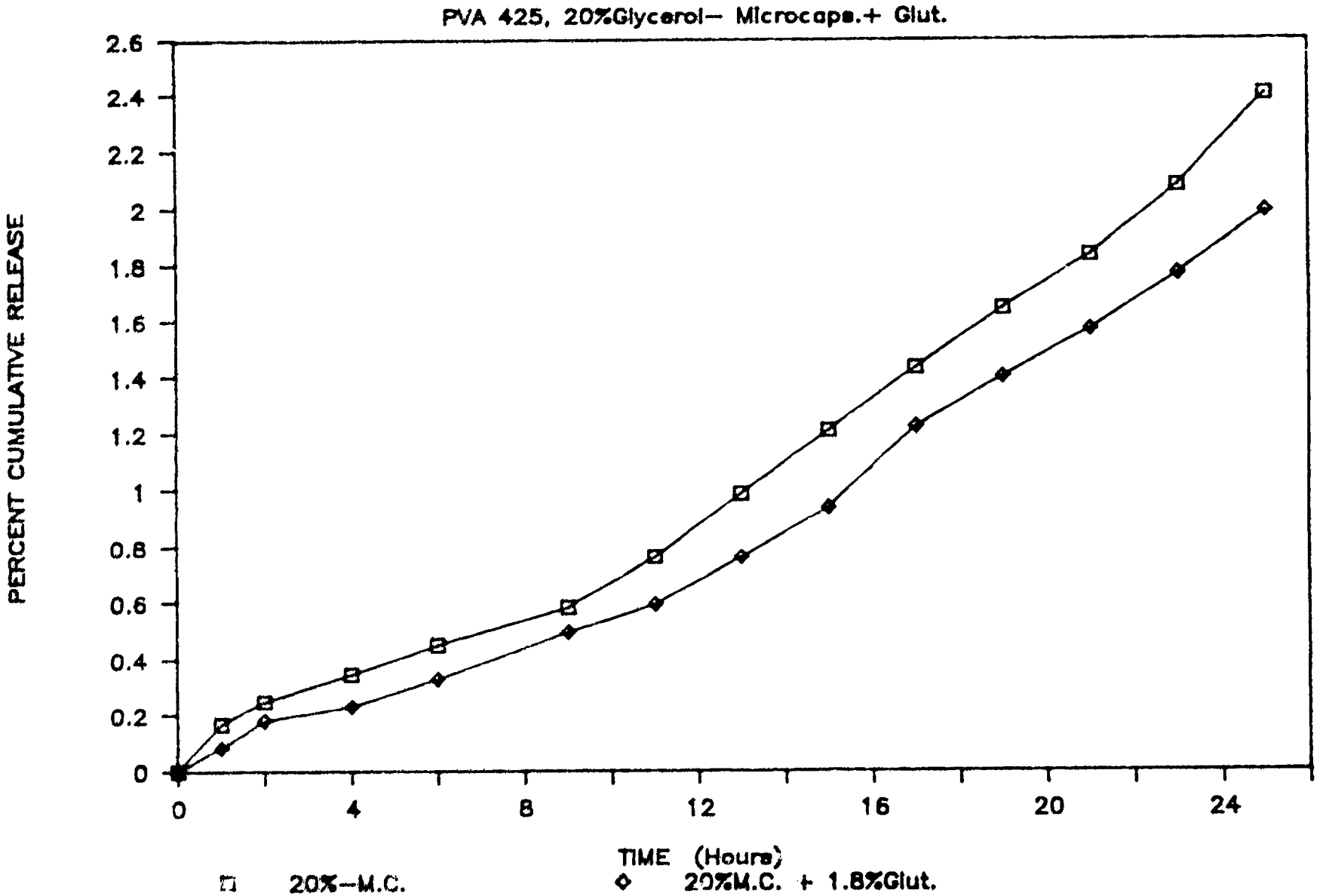


FIGURE 15

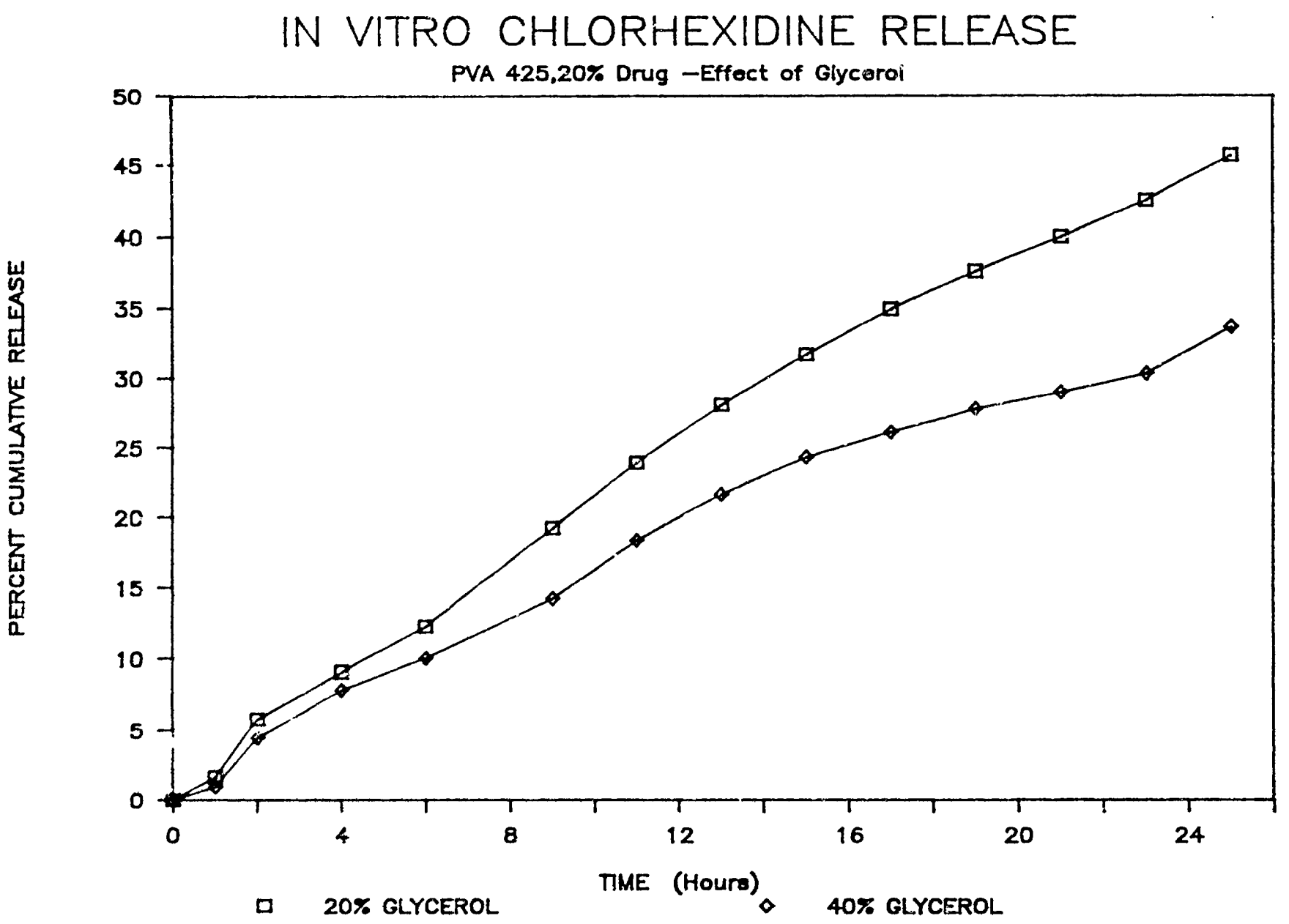

IN VITRO CHLORHEXIDINE RELEASE
PVA 425,20% Drug —Effect of Glycerol
PERCENT CUMULATIVE RELEASE
50
45
40
35
30
25
20
15
10
5
0
0
4
8
12
16
20
24
TIME (Hours)
20% GLYCEROL
40% GLYCEROL

FIGURE 16

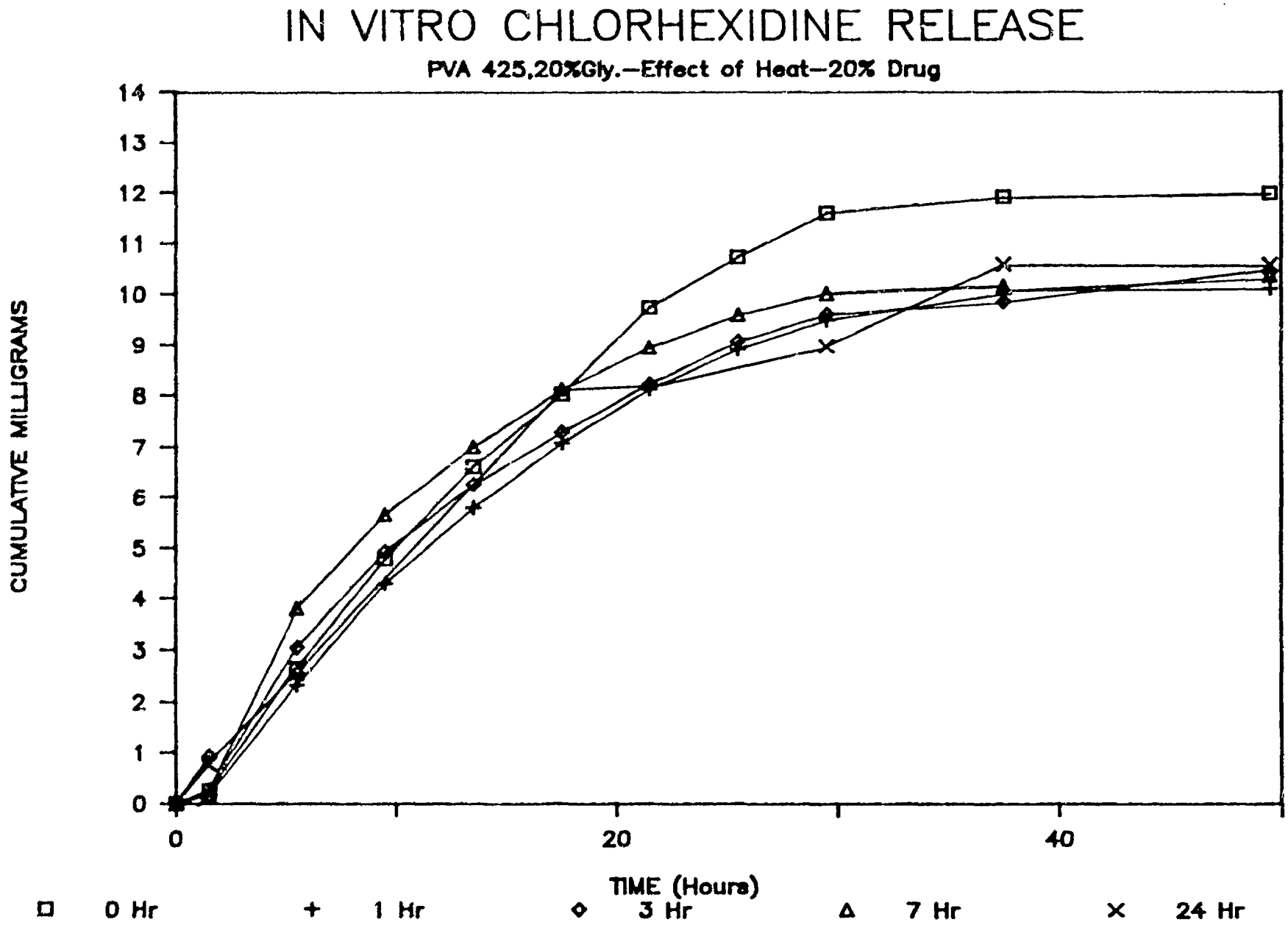

IN VITRO CHLORHEXIDINE RELEASE
PVA 425,20%Gly.—Effect of Heat—20% Drug
CUMULATIVE MILLIGRAMS
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0
20
40
TIME (Hours)
0 Hr
1 Hr
3 Hr
7 Hr
24 Hr

FIGURE 17

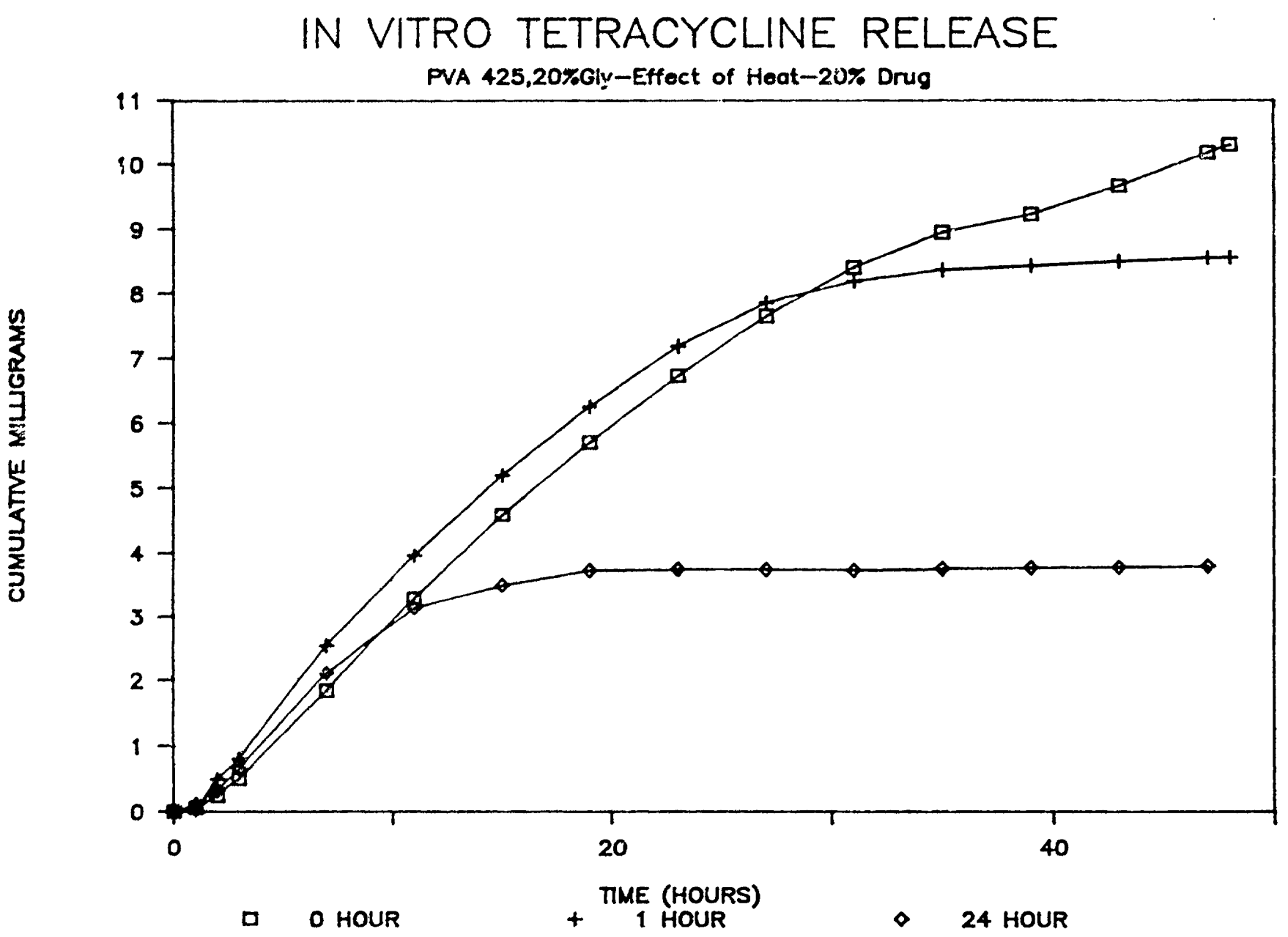

IN VITRO TETRACYCLINE RELEASE
PVA 425,20%Gly—Effect of Heat—20% Drug
CUMULATIVE MILLIGRAMS
11
10
9
8
7
6
5
4
3
2
1
0
0
20
40
TIME (HOURS)
0 HOUR
1 HOUR
24 HOUR

FIGURE 18

IN VITRO CHLORHEXIDINE RELEASE

PVA BLENDS –20% GLYCEROL –20% DRUG

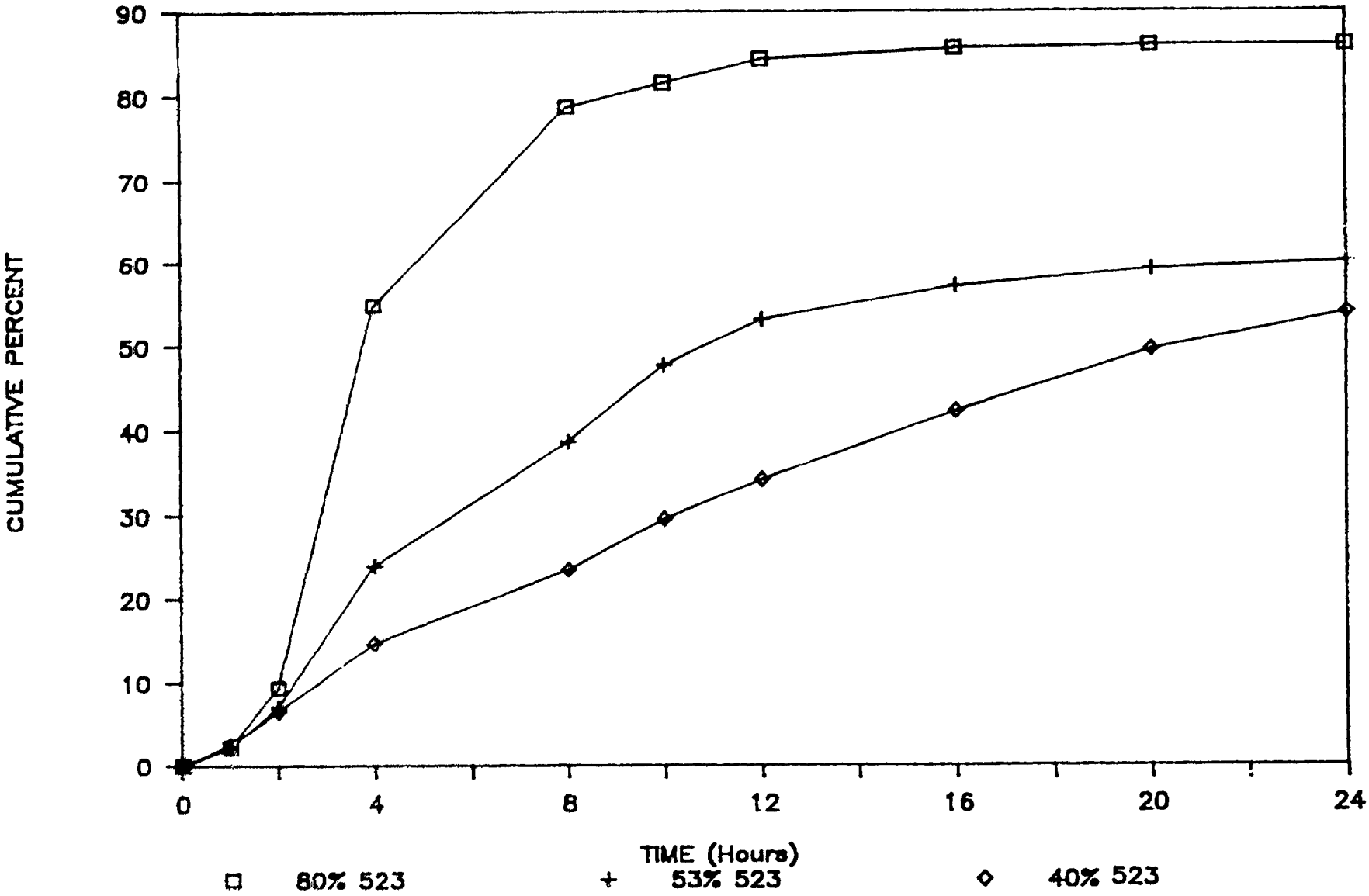


FIGURE 19

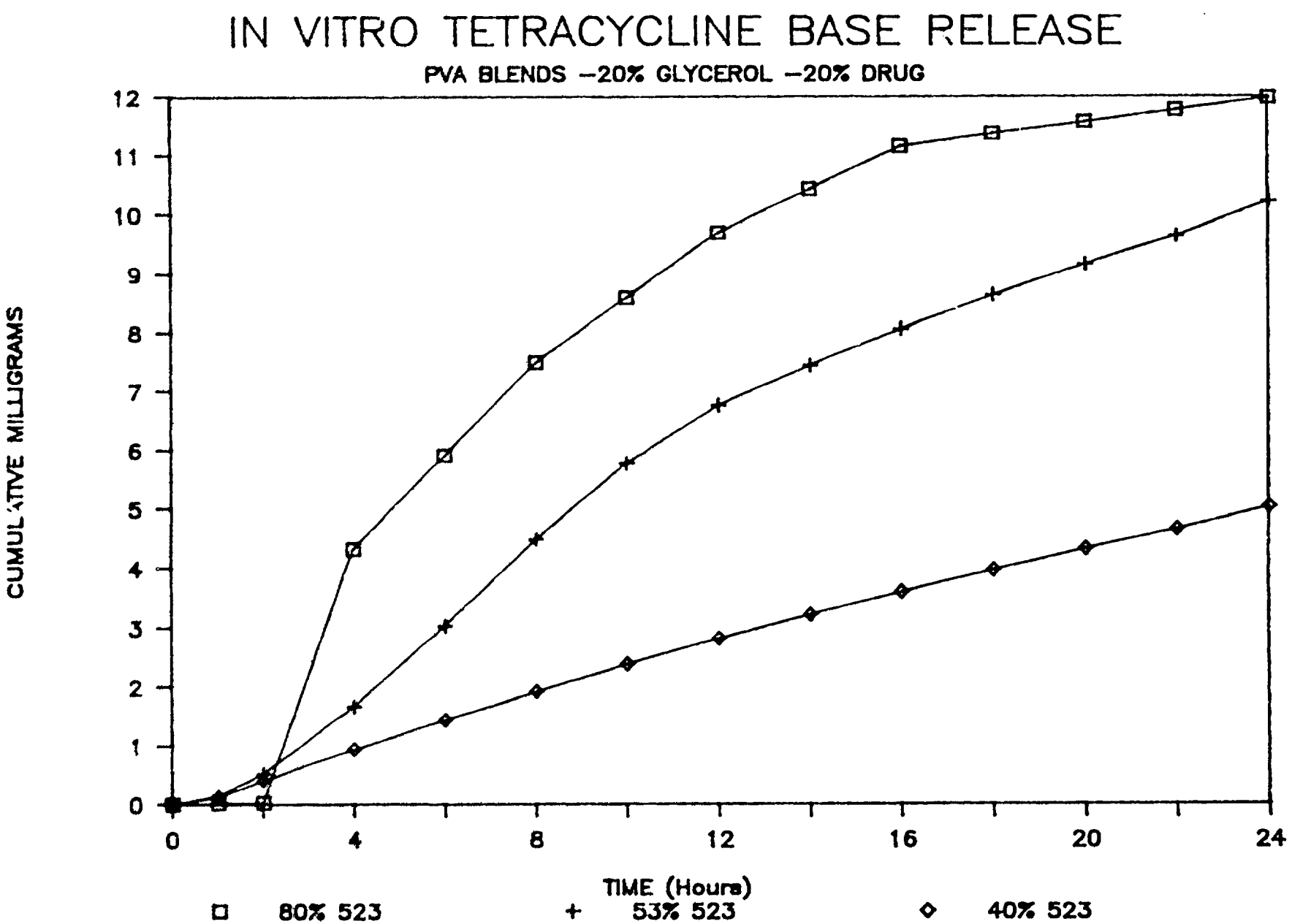

IN VITRO TETRACYCLINE BASE RELEASE
PVA BLENDS –20% GLYCEROL –20% DRUG
CUMULATIVE MILLIGRAMS
0
1
2
3
4
5
6
7
8
9
10
11
12
0
4
8
12
16
20
24
TIME (Hours)
80% 523
53% 523
40% 523

FIGURE 20

IN VITRO CHLORHEXIDINE RELEASE

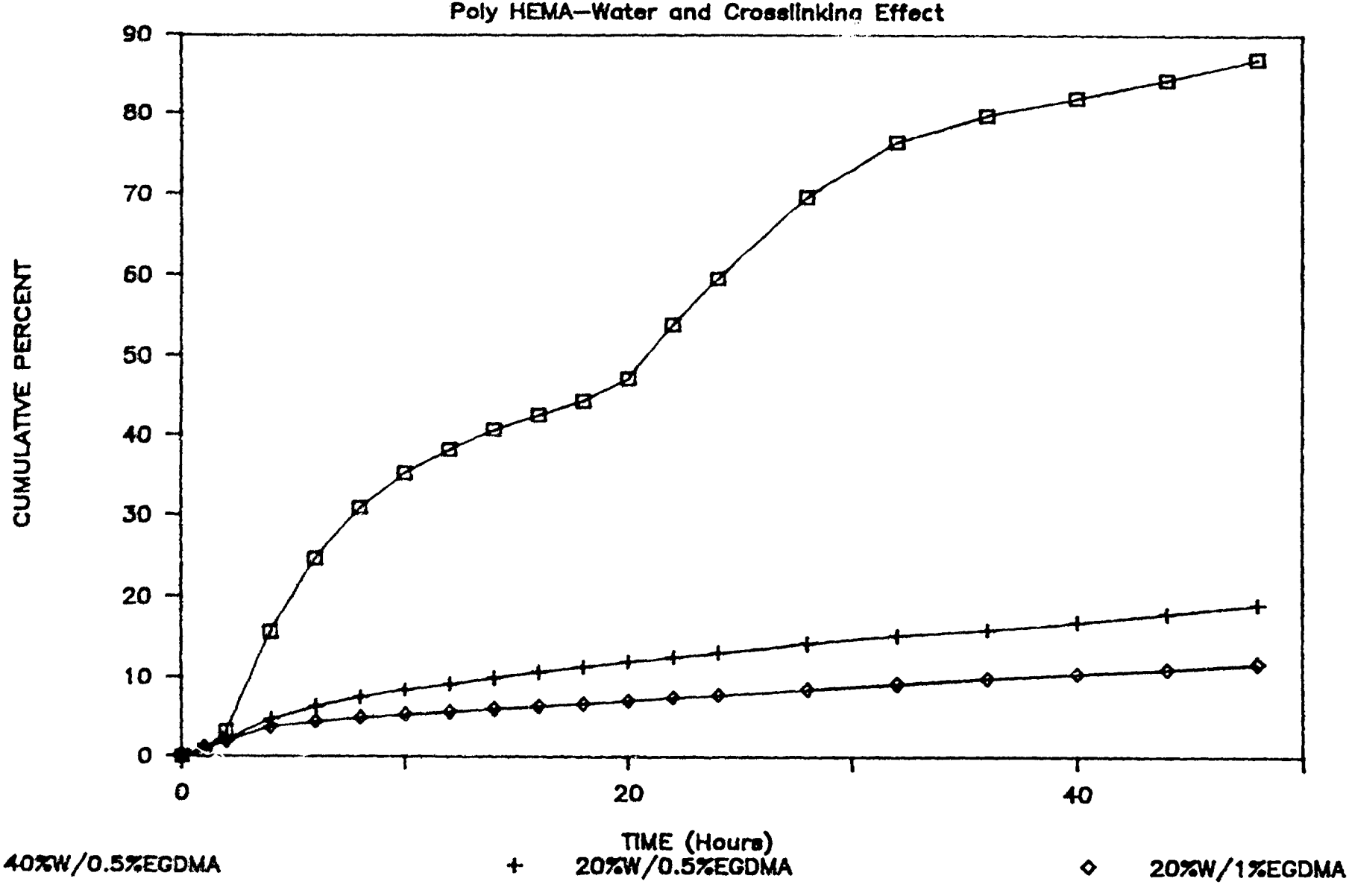


FIGURE 21

IN VITRO CHLORHEXIDINE RELEASE

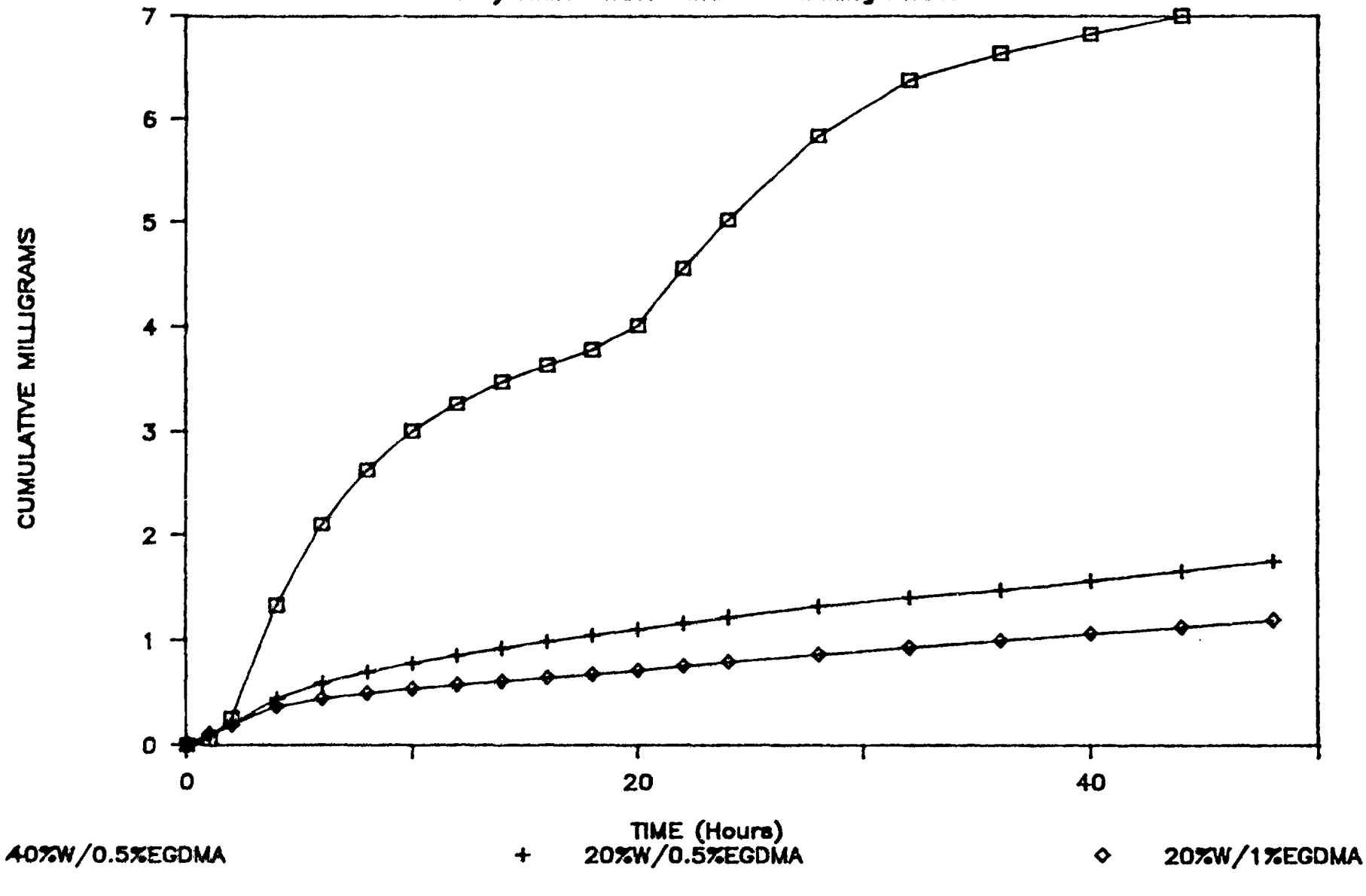


FIGURE 22

IN VITRO TETRACYCLINE BASE RELEASE

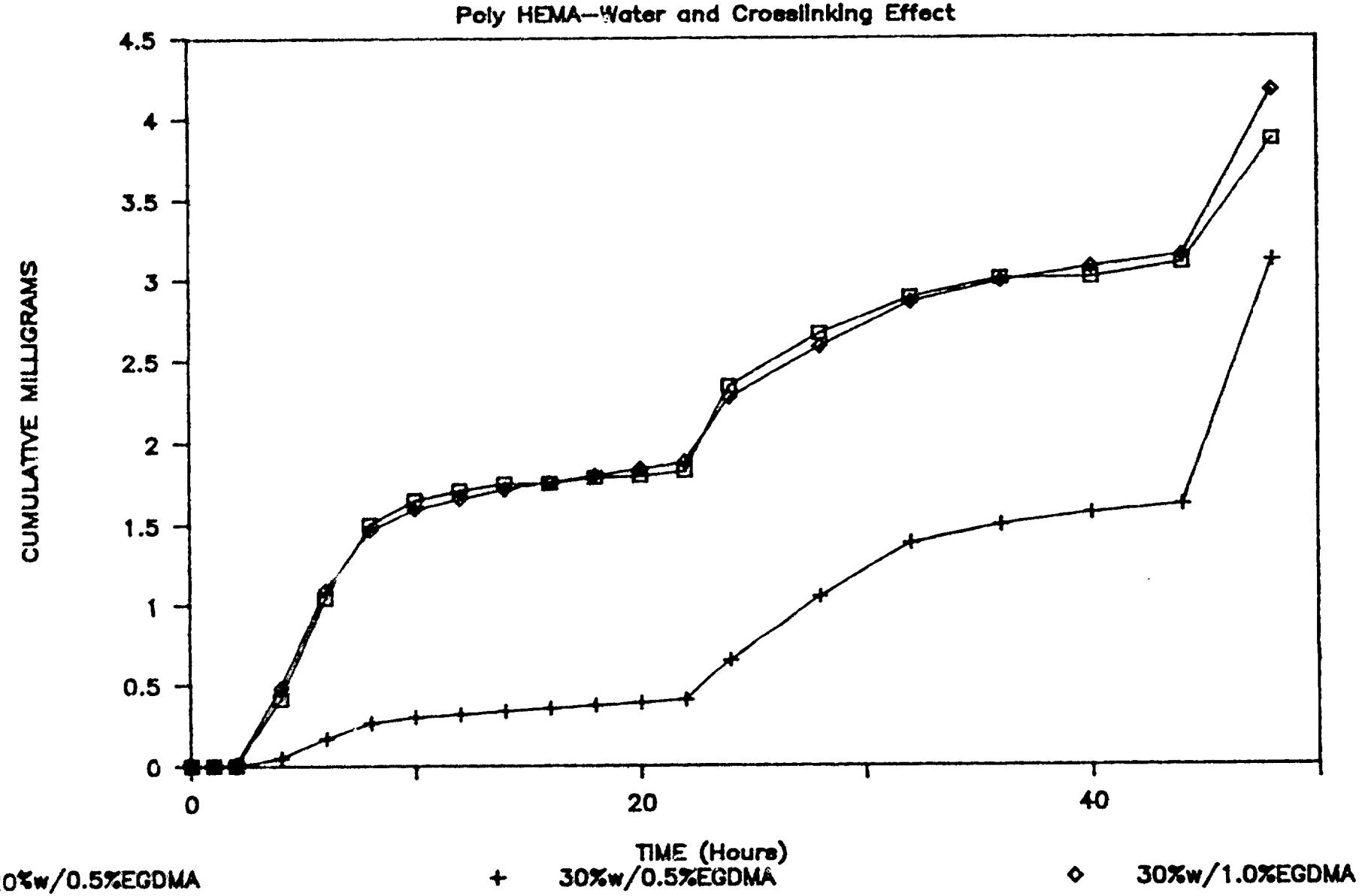


FIGURE 23

IN VITRO TETRACYCLINE BASE RELEASE

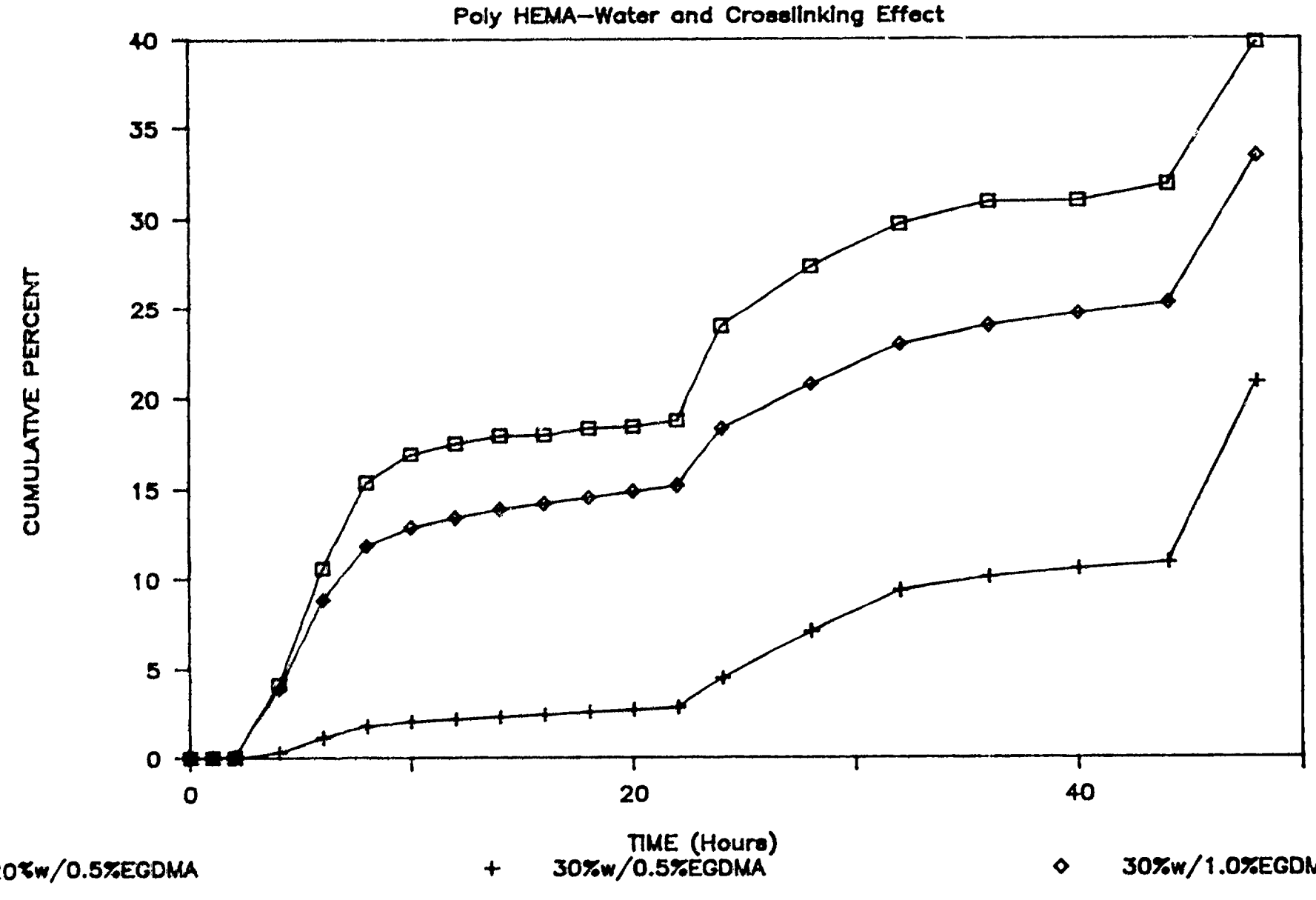


FIGURE 24

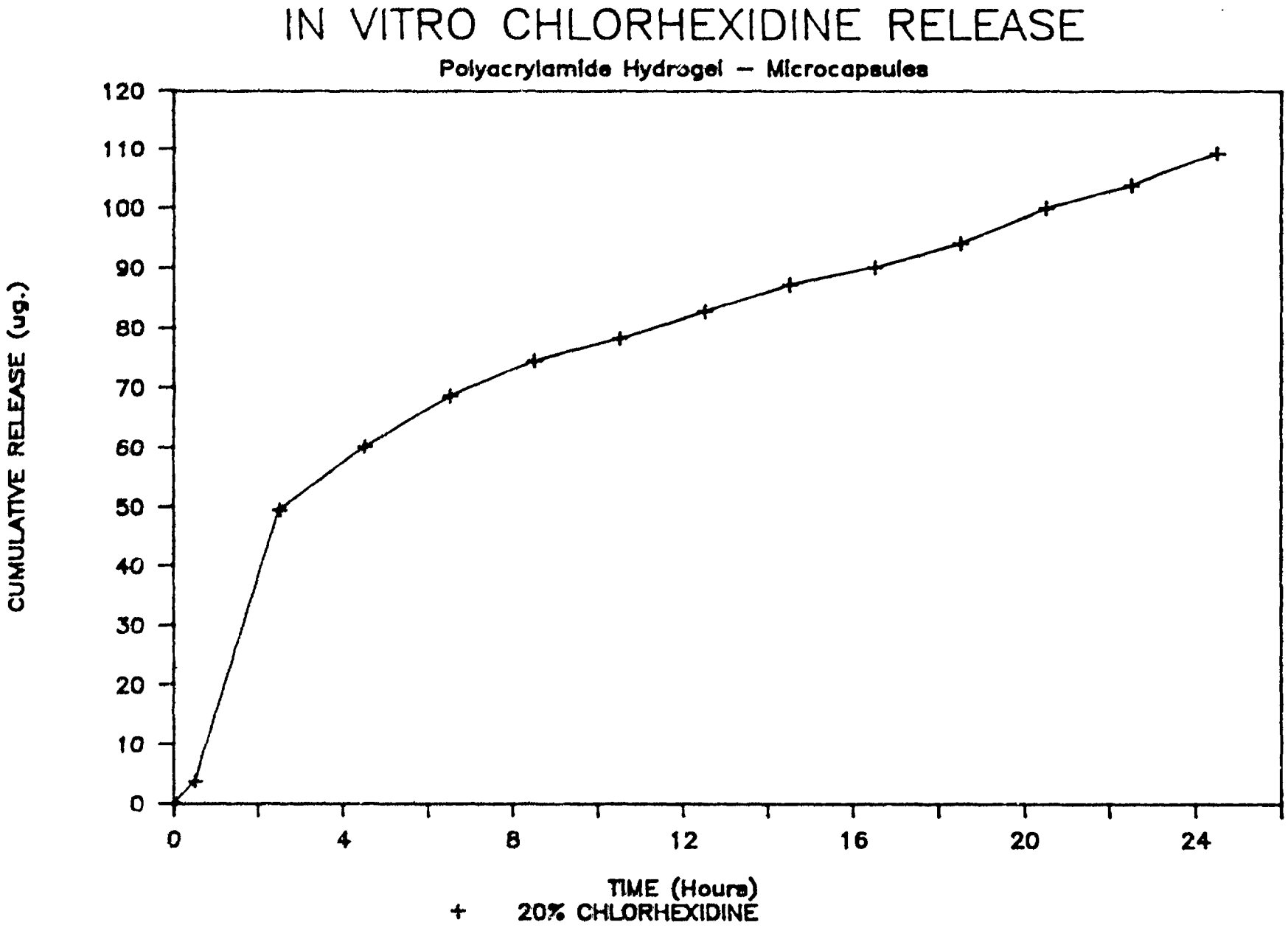

IN VITRO CHLORHEXIDINE RELEASE
Polyacrylamide Hydrogel – Microcapsules
CUMULATIVE RELEASE (ug.)
120
110
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
TIME (Hours)
+ 20% CHLORHEXIDINE

FIGURE 25

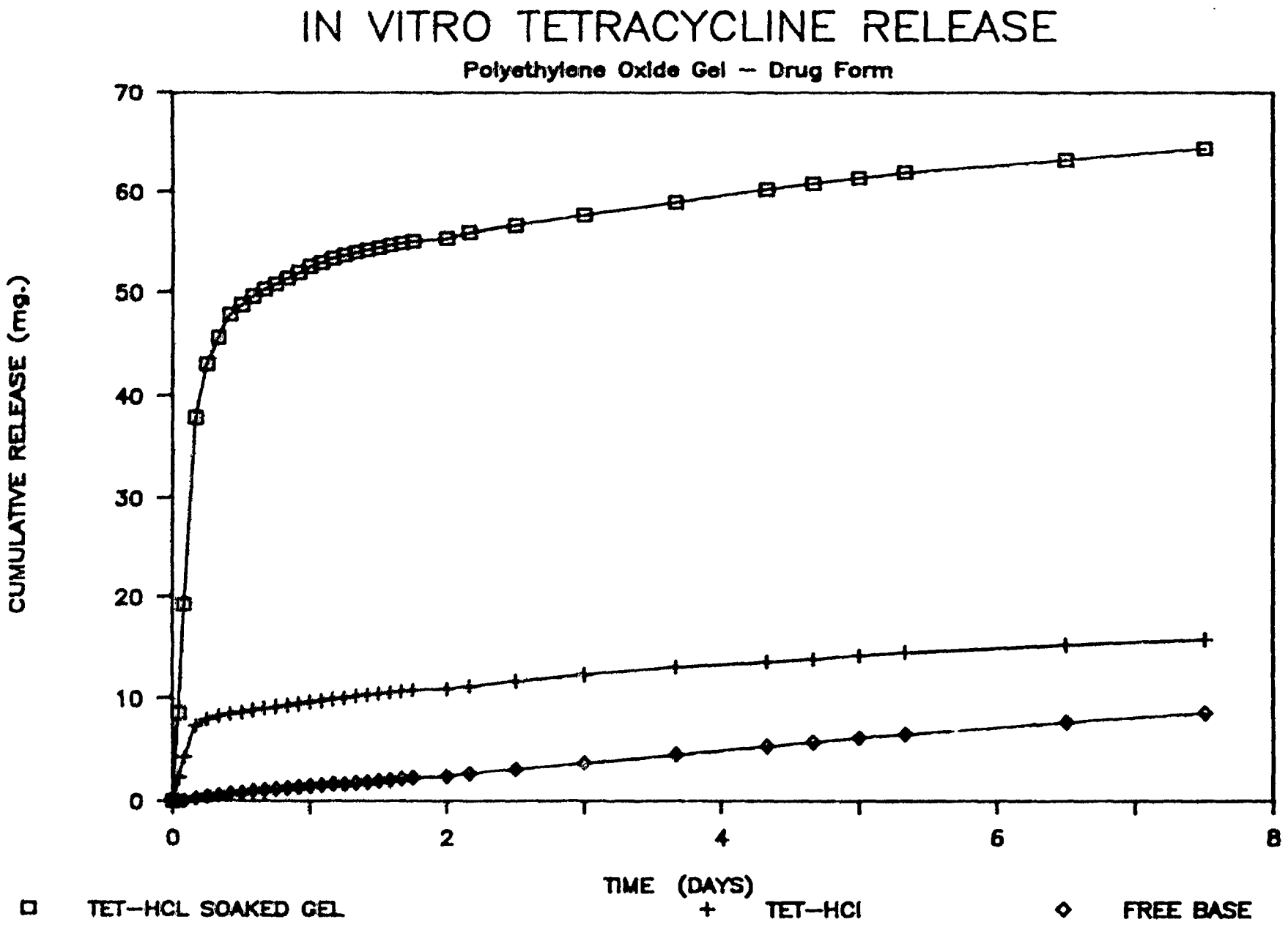
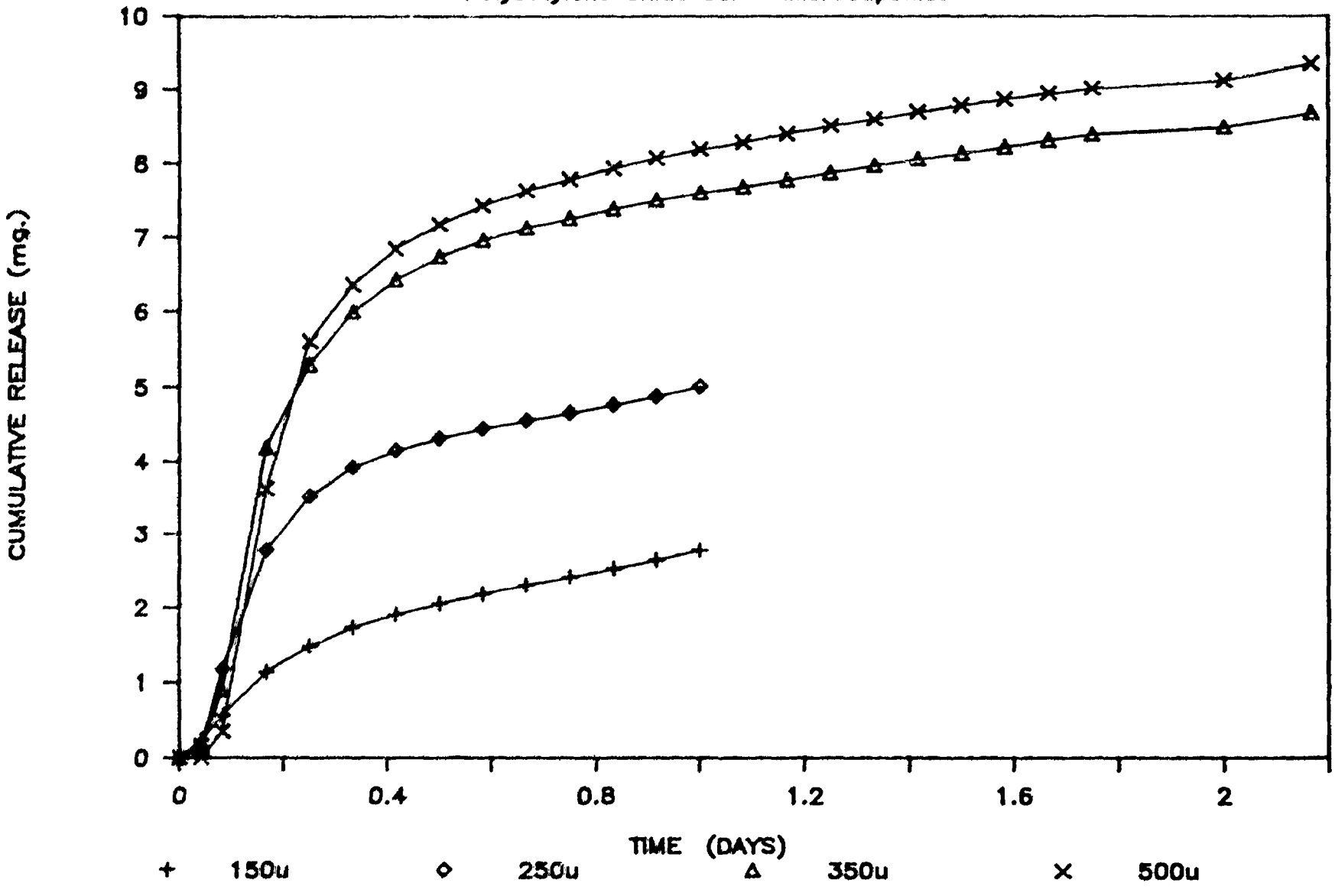

IN VITRO TETRACYCLINE RELEASE
Polyethylene Oxide Gel – Drug Form
CUMULATIVE RELEASE (mg.)
70
60
50
40
30
20
10
0
0
2
4
6
8
TIME (DAYS)
TET–HCL SOAKED GEL
TET–HCl
FREE BASE

FIGURE 26

IN VITRO TETRACYCLINE–HCl RELEASE



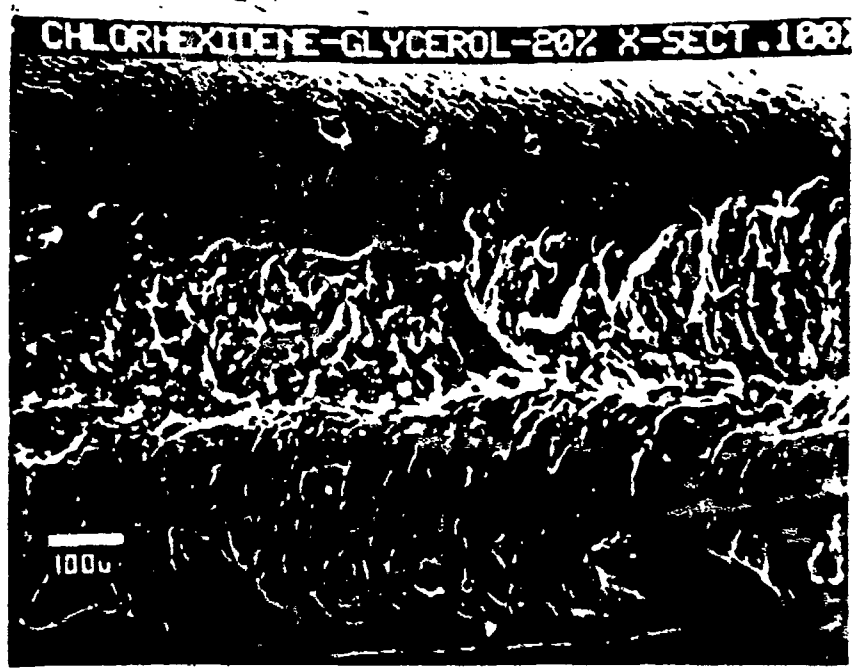


FIGURE 27 Cross Section Scanning Electron Micrograph of PVA Hydrogel. Matrix is composed of PVA 425 with 20% Glycerol as the Plasticizer. Chlorhexidine diphosphanilate concentration is 20%. 100X

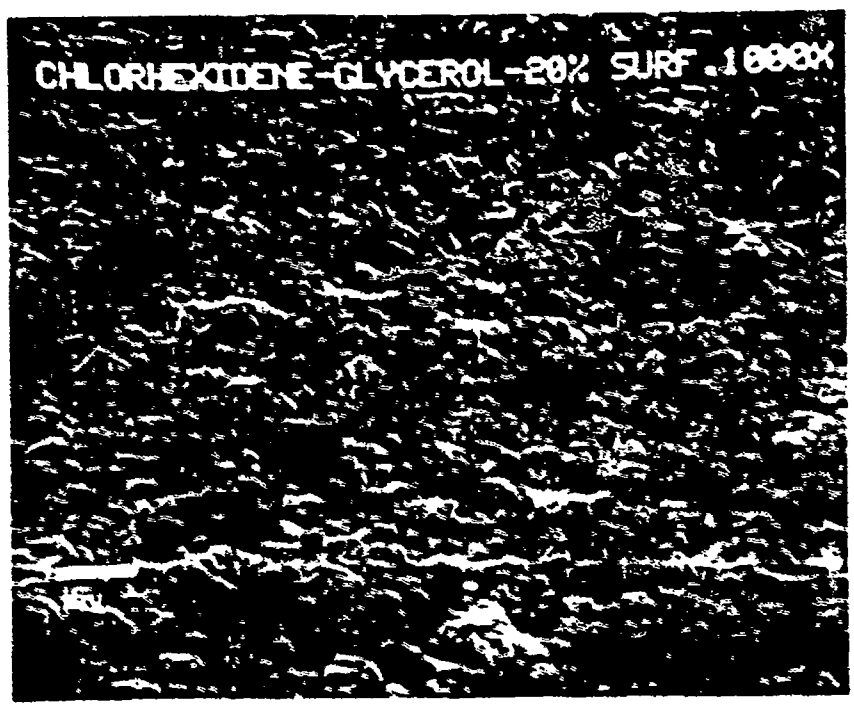


FIGURE 28 Surface Scanning Electron Micrograph of PVA Hydrogel. A surface view 1,000X of the same sample shown in Figure 27

FIGURE 29 Cross Section Scanning Electron Micrograph of Crosslinked PVA Hydrogel. PVA 425 with 20% blycerol was crosslinked with 2% glutaraldehyde. Chlorhexidine diphosphanilate concentration is 20%. 100X.

FIGURE 30 Surface Scanning Electron Micrograph of Crosslinked PVA Hydrogel. A surface view, 1,000X, of the same sample shown in Figure 29.